Clinical studies of the effect of food components on calcium or lipid metabolism(Dissertation_全文)

AUTHOR(S):

Fujiwara(Tomita), Kyoko

Clinical studies of the effect of food components on calcium or lipid metabolism

Kyoko Tomita-Fujiwara

2014

Contents

General Introduction

In recent years, the number of Japanese patients with lifestyle-related diseases such as diabetes, cardiovascular diseases, hypertension, osteoporosis, and cancer has been rapidly increasing; thus, such diseases have become a serious social concern. In 1984, the Japanese scientific academy proposed concept of tertiary function of food and defined “functional food” as a food having the tertiary or physiologically active function.[1)] That is, foods in general were defined to have 3 functions. The primary function was identified nutritional, that is essential for human survival. The secondary function was identified as sensory, or sensory satisfaction, such as “deliciousness”, flavor, and good texture. The tertiary function is physiological, such as regulation of the nervous system, the immune system, or body defense. After that, the Japanese Ministry of Health, Labour, and Welfare (MHLW) set up ‘Foods for Specified Health Use’ (FOSHU) in 1991 as a regulatory system to approve the statements made on food labels concerning the effect of the food on the human body.

Based on these background, various studies have examined the effects of several food ingredients on the human body, such as regulation of gastrointestinal conditions, anti-allergic effects, absorption of minerals, reduction of serum cholesterol, inhibitory effects on post-prandial levels of triglycerides, and anti-obesity effects.[2)] In addition, a number of “functional foods” were developed by applying the results of these studies. However, excessive commercialism has led to an increase in the number of products that have inaccurate efficacy claims listed on their label, as well as those that have not undergone proper safety testing. In 2003, the Health Promotion Act was revised to facilitate the regulation of these illegal products.

The current Japanese ‘‘Food with Health Claims’’ include 2 categories.[3)] For the first category, ‘‘Food with Nutrient Function Claims,’’ the label may be freely used if a product satisfies the standard for the minimum and maximum levels per daily portion usually consumed. The second category is defined as ‘‘Food for Specified Health Uses’’ (FOSHU). FOSHU foods are those that contain dietary ingredients that have beneficial effects on the physiological functions of the human

body, maintain and promote health, and improve health-related conditions.

When food products are submitted to FOSHU for approval, the Council of Pharmaceutical Affairs and Food Hygiene conducts scientific testing to evaluate their safety and efficacy. Once they have passed these evaluations, the food products are approved to label their efficacy (i.e., health claims). Mandated FOSHU documentation can be summarized into 3 essential requirements for FOSHU approval: 1) its effectiveness based on scientific evidence including clinical studies; 2) its safety as assessed from historical consumption pattern data and additional safety studies conducted in humans; and 3) analytical determination of the functional component responsible for the beneficial physiological action. The documentation of effectiveness should be prepared on the basis of substantiation, not only by human clinical and animal studies but by *in vitro* metabolism and biochemical data. [4)] As for safety, the several studies *in vitro* and in animals were required in order to obtain the preliminary data confirming the food's safe intake by human. Even if an effective component has been consumed as food by a reasonable number of individuals during a certain period, safety data for human consumers also should be required. [5-7)] From the perspective of the manufacturer, it is desirable to be able to emphasize the characteristics of their products and promote them using the labeled health claims. On the other hand, significant time and financial resources are required for the research and development needed to gain approval as FOSHU.

It should be also considered that FOSHU are designed to target healthy people or people in a preliminary stage of a disease or a borderline condition of at-risk groups. Also all foods including "functional foods" can be ingested freely by all people from children to elderly without the directions for use. Therefore, it is critical for manufacturers to strictly evaluate both the efficacy and safety of their food products, as well as the ingredients they contain.

As mentioned earlier, the labeling of health claims for foods should always be based on rigorous scientific testing. In this study, the author has summarized various studies of several food ingredients with health-promoting potency, which were aimed to be developed as "functional foods" with labelled health claims. Chapter 1 reveals that short-term ingestion of (−)-hydroxycitrate (HCA), an

active ingredient extracted from the rind of the fruit of *Garcinia cambogia*, increased fat oxidation in untrained men. Chapter 2 elucidates that ingestion of nondigestible disaccharide difructose anhydride III (DFAIII) enhanced intestinal calcium absorption in a human balance study. Chapter 3 suggests how dietary melibiose affected the T helper (Th) cell responses induced by an orally fed antigen in ovalbumin (OVA)-specific T cell receptor transgenic mice (OVA 23-3). Furthermore, Chapter 4 confirms that apple polyphenol (AP) had no toxicological effects in the 90-day oral feeding test of rats and could be safely used as food for human consumers.

References

1) Arai, S., Studies on functional foods in Japan. *Biosci. Biotech. Biochem.*, 60, 9-15 (1996).
2) Arai, S., Morinaga, Y., Yoshikawa, T., Ichiishi, E., Kiso, Y., Yamazaki, M., Morotomi, M., Shimizu, M., Kuwata, T., and Kaminogawa, S., Recent trends in functional food science and the industry in Japan. *Biosci. Biotech. Biochem.*, 66, 2017-2029 (2002).
3) Director General, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labor and Welfare, Creation of the food with health claims system. Iyakuhatsu Notification No.244, March 27 (2001) (in Japanese).
4) Morinaga, Y., Shimizu, T., Sueki, K., and Hirahara, T., *Health Claim on Functional Foods.* Japan; International Life Scientific of Japan (2000).
5) Shimizu, T., Health claims on functional foods: the Japanese regulations and an intestinal comparison. *Nutr. Res. Rev.*,16, 241-252 (2003).
6) Ohama, H., Ikeda, H., Moriyama, H., Health foods and foods with health claims in Japan. Toxicology, 221, 95-111 (2006).
7) Yamada K., Sato-Mito, N., Nagata, J., Umegaki, K., Healht claim evidence requirements in Japan. *J. Nutr.*, 138, 1192-1198 (2008).

Chapter 1

(－)-Hydroxycitrate ingestion increases fat oxidation during moderate intensity exercise in untrained men

(－)-Hydroxycitrate (HCA), an active ingredient extracted from the rind of the fruit of *Garcinia cambogia*,[1] is a potent competitive inhibitor of ATP-citrate lyase (EC 4.1.3.8).[2] Recently, HCA has been used for anti-obesity treatment and the clinical relevance of HCA has often been proposed.[3–5] There are also some reports examined the effects of HCA ingestion on endurance exercise performance in rats[6] or human athletes.[7] Thus, HCA supplementation with some exercise could be useful as an ergogenic aid increases fat oxidative capacity and is further expected to be a metabolic anti-obesity agent. However, energy utilization systems during exercise are different with or without a training state. In this study, we examined the effects of short-term HCA ingestion on fat oxidation during moderate intensity exercise in ordinary untrained men.

Six untrained males participated in the study. They did not take any regular exercise training for at least 6 months. They did not control their food consumption or private life style during the experimental periods. Their physical characteristics were as follows: 20.2±1.5 years old, 177.0±3.4 cm tall, 69.7±7.2 kg weigh, and 16.5±3.8% of body fat. This study was approved by Kyung Hee University in Korea, in accordance with the Helsinki Declaration of 1975. Subjects' mean incremental maximum oxygen consumption (VO_{2max}) measured on a bicycle ergometer was 43.0± 5.6 ml/kg^{-1}/min^{-1}. We calculated their exact 40% and 60% of VO_{2max} excise bout based on their VO_{2max} and divided them into two groups. They ingested HCA or placebo for 5 days in a double-blind and crossover manner. At least 2 days between the trials was established to minimize any possible effects of HCA. HCA was taken as 50 ml of drinks containing 500 mg of HCA. Placebo did not contain HCA but had the same amount of the energy and carbohydrate, protein, and fat was adjusted to the same as that of HCA. HCA used in this study was as soluble types and provided by Nippon Shinyaku (Kyoto, Japan).

The experimental design is shown in Fig. 1. On the 5th day, subjects reported and ingested 620 kcal of the meal and HCA or Placebo. Then, subjects started to warm up and exercise using a bicycle ergometer (Combi Aerobike, 75TXL-2, Japan) at a pedalling frequency of 50 rpm and an intensity of 40% of VO_{2max} for 60 min, and then the intensity was elevated to 60% until they were exhausted. The two different exercise-intensity exercises protocol was selected to investigate endurance performance as reported by some researchers.[7–9] Expired gas samples and blood samples were collected. Exercise time to exhaustion was measured when the cadence could no longer be maintained three times under 50 rpm. The experiments were done with a room temperature of 20°C and 50% humidity. Expired gas samples were analyzed on an auto-analyzer (Sensor Medic, Vmax 229, USA). Fat and carbohydrate oxidation during exercise was calculated as previously described.[10] Blood glucose and lactate concentrations were analyzed using an auto-analysis system and plasma free fatty acid (FFA) concentrations were measured by an enzymatic method using a kit.[7,8] The results are described as mean±SE. To compare the data, a paired *t*-test was done between trials. Significant differences between values were measured using JSTAT (JSTAT 9.0 for windows). The level of significance was set at $p < 0.05$.

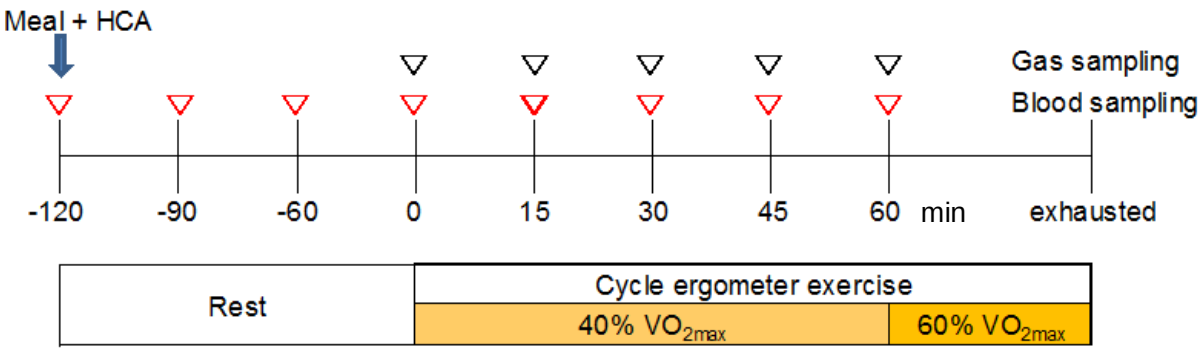

Fig. 1. Experimental Design (5th day)

The experimental results are as follows. Oxygen consumption (VO_2) was not different between the HCA and placebo ingestion groups (Fig. 2A). On the other hand, the respiratory exchange ratio (RER) in the HCA ingestion group significantly decreased compared with the placebo group ($p < 0.05$) (Fig. 2B). Also, fat oxidation in HCA trials increased from 30 min of the exercise, significantly at 45 min of the exercise ($p < 0.05$) while carbohydrate oxidation tended to decrease during the same period (data not shown). Endurance exercise training reduces the malonyl-CoA concentration[11] and increases the mitochondrial contents,[12] and also increases the mitochondrial uptake of long-chain fatty acids.[13] That is why fat oxidation is increased. These results suggested that HCA ingestion increases the fat oxidation capacity and spares carbohydrate use during the same-intensity exercise even in untrained men. Blood FFA concentration during exercise time increased in the HCA ingestion group, significantly at 15 min and 60 min of the exercise ($p < 0.05$) (Fig. 3). This also suggested that HCA ingestion increased fatty acids use as an energy source during moderate intensity exercise. Increased FFA concentration might have a glycogen sparing effect and there are some reports that blood lactate levels during exercise were slightly decreased by HCA ingestion.[7,14] However, glucose and lactate concentrations were not changed in this study (data not shown). It is considered that the effects of HCA on lactate levels may be affected by the subjects' training state, but the details remain unclear. And also, exercise times to exhaustion at 60% VO_{2max} after 40% VO_{2max} for 1 h were slightly prolonged by HCA ingestion but the differences were not significant (data not shown). Thus, the effects of HCA ingestion on endurance exercise capacity were not clear. Kriketos *et al.*[15] failed to detect an effect of short term HCA ingestion on lipid oxidation during moderate intensity exercise in men. The experimental design was very similar to this study, but the subjects ingested 3.0 g of insoluble HCA just for 3 days, and the endurance exercise time was shorter. There are also some reports that an acute administration of HCA did not affect energy substrate use.[6,11,14] It can be predicted that some continuous ingestion term is needed to detect the effects of HCA on fat oxidation and the ingestion form of HCA or exercise time could be the important factor for these experiments.

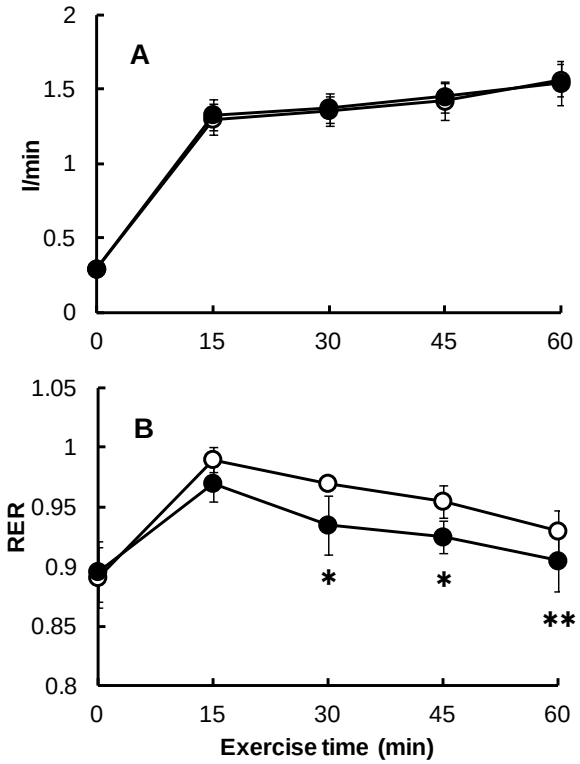

Fig. 2. VO_2 during Exercise (A) and Respiratory Exchange Ratio (B) during Exercise at 40% VO_{2max}.

Values are means ± SE. Statistically significant from the values of the placebo trials (*p < 0.05, **p <0.01)

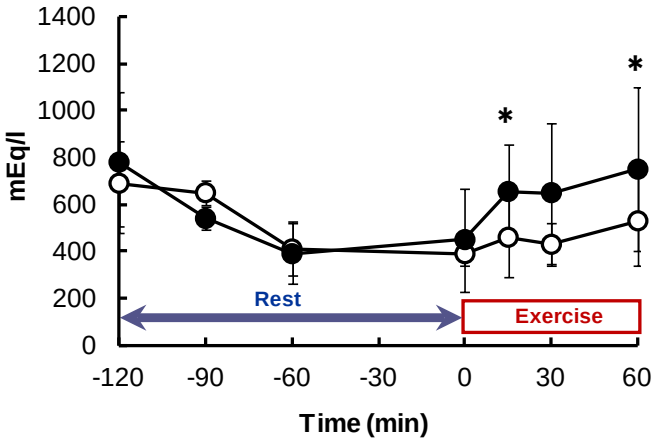

Fig. 3. Changes of Blood FFA Concentrations throughout the Trials.

Values are means ± SE. Statistically significant from the values of the placebo trials (*p < 0.05)

In conclusion, it was seen that short-term ingestion of HCA increases fat oxidation during moderate intensity exercise also in untrained men.

References

1) Lewis, Y. S., and Neelakantan, S., (-)-Hydroxycitric acid—the principal acid in the fruits of Garcinia cambogia. *Phytochemistry*, 4, 610–625 (1965).

2) Watson, J. A., Fang, M., and Lowenstein, J. M., Tricarballylate and hydroxycitrate: substrate and inhibitor of ATP: citrate oxaloacetate lyase. *Arch. Biochem. Biophys*., 135, 209–217 (1969).

3) Badmaev, V., Majeed, M., and Conte, A. A., Garicinia cambogia for weight loss. *JAMA*, 282, 233–234, discussion 235 (1999).

4) Schaller, J. L., Garicinia cambogia for weight loss. *JAMA*, 282, 234, discussion 235 (1999).

5) Hayamizu, K., Ishii, Y., Kaneko, I., Shen, M., Sakaguchi, H., Okuhara, Y., Shigematsu, N., Miyazaki, S., and Shimasaki, H., Effects of longterm administration of Garcinia cambogia extract on visceral fat accumulation in humans: a placebocontrolled double blind trial. *J. Oleo Sci.* (in Japanese), 50, 805–812 (2001).

6) Ishihara, K., Oyaizu, S., Onuki, K., Lim, K., and Fushiki, T., Chronic (-)-hydroxycitrate administration spares carbohydrate utilization and promotes lipid oxidation during exercise in mice. *J. Nutr.*, 130, 2990–2995 (2000).

7) Lim, K., Ryu, S., Ohishi, Y., Tomi, H., Suh, H., Lee, W.-K., and Kwon, T., Short-term (-)-hydroxycitrate ingestion increases fat oxidation during exercise in athletes. *J. Nutr. Sci. Vitaminol.*, 48, 128–133 (2002).

8) Ryu, S., Choi, S.-K., Joung, S.-S., Suh, H., Cha, Y.- S., Lee, S., and Lim, K., Caffeine as a lipolytic food component increases endurance performance in rats and athletes. *J. Nutr. Sci. Vitaminol*., 47, 139–146 (2001).

9) Lim, K., Yoshioka, M., Kikuzato, S., Kiyonaga, A., Tanaka, H., Shindo,M., and Suzuki,M., Dietary red pepper ingestion increases carbohydrate oxidation at rest and during exercise in runners. *Med. Sci. Sports Exerc*., 29, 355–361 (1997).

10) Angus, D. J., Hargreaves, M., Dancey, J., and Febbraio,M., Effect of carbohydrate or carbohydrate plus medium-chain triglyceride ingestion on cycling time trial performance. *J. Appl. Physiol*., 88, 113–119 (2000).

11) Hutber, C. A., Rasmussen, B. B., and Winder, W. W., Endurance training attenuates the decrease in skeletal muscle malonyl-CoA with exercise. *J. Appl. Physiol*., 83, 1917–1922 (1997).

12) Constable, S. H., Favier, R. J., McLane, J. A., Fell, R. D., Chen, D., and Holloszy, J. O., Energy metabolism in contracting rat skeletal muscle: adaptation to exercise training. *Am. J. Physiol*., 253, C316–C322 (1987).

13) Sidossis, L. S., Gastaldelli, A., Klein, S., and Wolfe, R. R., Regulation of plasma fatty acid oxidation during low- and high-intensity exercise. *Am. J. Physiol*., 272, E1065–E1070 (1997).

14) Van Loon, L. J., Van Rooijen, J. J., Niesen, B., Verhagen, H., Saris, W. H., and Wagenmakers, A. J., Effects of acute (－)-hydroxycitrate supplementation on substrate metabolism at rest and during exercise in humans. *Am. J. Clin. Nutr*., 72, 1445–1450 (2000).

15) Kriketos, A. D., Thompson, H. R., Greene, H., and Hill, J. O., (－)-Hydroxycitric acid does not affect energy expenditure and substrate oxidation in adult males in a post-absorptive state. *Int. J. Obes. Relat. Metab. Disord*., 23, 867–873 (1999).

Chapter 2

Ingestion of difructose anhydride III enhances absorption and retention of calcium in healthy men

Calcium is an essential nutrient, and adequate calcium intake is recommended for the development of high peak bone mass and the prevention of osteoporosis.[1)] To improve nutritional status as to calcium, increases in intake are important, but intestinal calcium absorption is affected by various factors, such as the source of supplied calcium[2,3)] and co-ingested food components.[4)] The ability of calcium absorption is also influenced by age.[5)] Therefore, it is necessary to consider the absorptive efficacy of ingested calcium together with adequate intake.

Several nondigestible carbohydrates, such as fructooligosaccharides, have been reported to promote calcium absorption in *in vivo* studies.[6–10)] Difructose anhydride III (DFAIII; di-D-fructofuranose-1,2':2,3'-dianhydride) has also been shown to enhance calcium absorption in both the small and the large intestine of rats.[11–18)] DFAIII is a naturally occurring nondigestible disaccharide in the root of *Lycoris radiate*,[19)] and is also found in caramels[20,21)] and roasted chicory.[22)] This disaccharide has recently been manufactured from inulin with *Arthrobacter* sp. H65-7 fructosyltransferase[23,24)] and is used as a food material. The mechanisms of promotion of calcium absorption with DFAIII have been identified as follows: (i) intact DFAIII stimulates paracellular calcium absorption in the small intestine, and (ii) DFAIII increases large intestine calcium absorption *via* short-chain fatty acid (SCFA) production.

The aim of the present study was to examine the effects of DFAIII ingestion on calcium absorption in human subjects. In a previous study, we observed temporal increases in urinary calcium excretion after an oral load of calcium with DFAIII, which suggests stimulation of DFAIII on intestinal calcium absorption in humans.[25)] However, this method was based on a positive correlation between intestinal calcium absorption and urinary calcium excretion in the short term,

and results were affected by renal clearance of calcium. In the present study, we adopted a balance study in human subjects to examine the effects of DFAIII ingestion on calcium absorption.

Material and Methods

Test food and control food. DFAIII (>97%) was obtained from Nippon Beet Sugar (Tokyo). The test foods were composed of 1.0 g of DFAIII and 250 mg of scallop shell powder as calcium carbonate (corresponding to 100 mg of calcium). Control foods were composed of 250 mg of folded scallop shell powder only.

Subjects. We recruited volunteers. Twenty healthy males agreed to participate in this study. The protocol complied with the Helsinki Declaration and received approval from the ethics committees of the Fancl Corporation. We informed all participants of the purpose and plan of this study and of their right to resign at any time of their own free will, and of our duty to guard their personal privacy against outsiders. All subjects satisfied the following conditions: (i) No history of daily intake of calcium-enriched foods or health foods to stimulate calcium absorption. (ii) No intake of vitamin D, vitamin K, or other drugs to treat or prevent osteoporosis, and no agents mediating bone metabolism. (iii) No history of bone-related disorders, diabetes, or other hepatic or renal function disorders. We finally selected 14 males by fecal condition and their volume.

Study design. The study was designed in a single-blind, crossover manner. The 14 subjects were divided into two groups. They ingested the test food or the control food three times a day before each meal for 13 d. The whole feces and urine were collected for the last 4 d (day 10–day 13) as a balance period. The subjects repeated the 13-d ingestion period with another test food after an 8-day washout period. The experimental schedule is illustrated in Fig. 1.

On the first day of the experiment (day 1), the subjects underwent a physical examination and health interviews with a physician, and blood and urine samples were collected for the initial value.

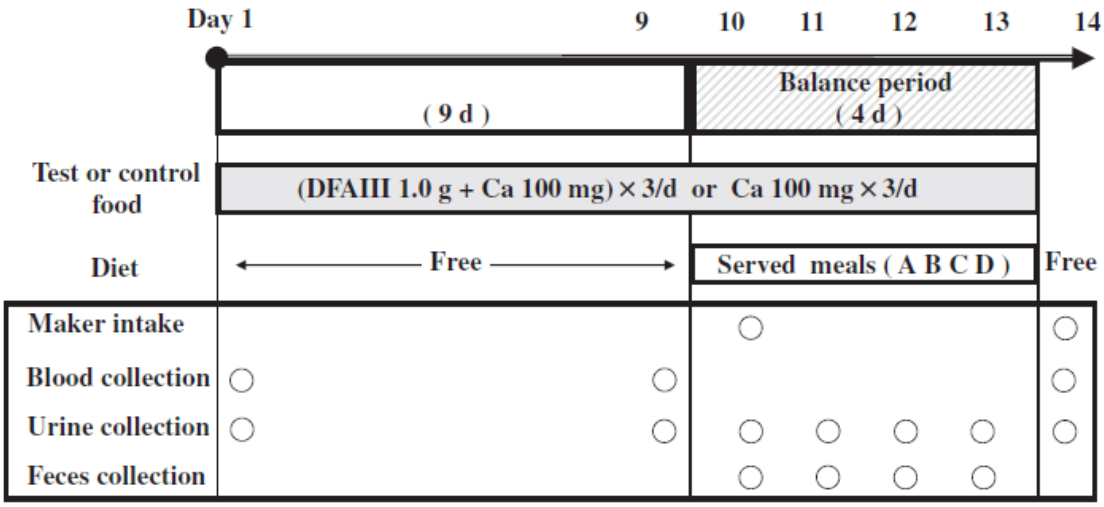

Fig. 1. Experimental Schedule.

Subjects ingested calcium or calcium and DFAIII for 13 d. After the first 9 d, the balance study was performed.

The subjects were instructed to record their overall physical condition, stool frequency and condition, and the presence and severity of specific abdominal or any other symptoms every day on a specified log sheet. They did not have any dietary restriction during the first 9 d of the experimental period, without taking test food. The nutrient contents of their diets were analyzed for 3 d of this period by the previously reported method,[26] taking photographs of their meals with a camera equipped with a personal digital assistant (PDA). The data were sent to dietitians via an attached mobile phone card for calculation of the nutrients using computer software (Wellnavi; Matsushita Electric Works, Osaka, Japan). From day 9, 1 d before the start of a balance period, to day 13, the subjects were kept in the hospital under a unified protocol with restrictions on movement out of the hospital, and abstinence from both alcohol and excessive exercise. They were served meals to control nutrition intake during the balance period. Four different meal sets were prepared for each experimental day. Each subject had breakfast at 9:00, lunch at 12:00, and dinner at 19:00. The menu contents and the nutrition compositions of the meals are shown in Table 1.

On day 10, subjects collected their urine before breakfast and ingested 0.5 g of coloring marker (carmine, Sigma, St. Louis, MO) with 50 ml of water to determine the start line of fecal sampling,

then ingested the test food or control food with 100ml of water (narural mineral water, Suntoryfoods, Tokyo). The subjects were allowed to drink this freely (recording the amount by themselves), and 24-h total urine and all fecal samples were collected for 4 d. The 4 d of collected urine for each subject was pooled, weighed, and stored at -20 ˚C until analysis. Feces were collected in plastic bottles, freeze-dried, and stored at -20 ˚C until analysis. Blood sample were also stored at -20 ˚C. On day 14 (the first day of the washout period), the subjects again ingested 0.5 mg of carmine to determine the end line of feces sampling and underwent a physical examination and health interviews, and blood samples were collected.

Table 1. Four Sets of Served Meals

	A	B	C	D
Breakfast	Boiled rice Miso soup Pork saute Namul (vegetable marinated with sesame oil) Fry boiled down in soy sauce	Bread Jam Tuna salad Fruit	Boiled rice Miso soup Grilled fish Boiled vegetables Steamed fish paste Roasted laver	Boiled rice Miso soup Fermented soybeans Pickles Bolied dried-radish strips
Lunch	Festive red rice Flied shrimp Spaghetti with neapolitan sauce Tartar sauce Sauteed vegetables Salad Pickles	Boiled rice Sliced beef boiled with vegetables Sauteed onion and spaghetti Food boiled down in soy souse Cooked fried-bean curd and konnyaku Fruit	Boiled rice Pork cutlet with curry Macaroni salad Fruit Pickles	Boiled rice Meuniere fish Pickles Food boiled down in soy souse Potato salad Fruit
Dinner	Boiled rice Grilled fish Grated radish Boiled spinach Tomato Simmered meat and potatoes White radish sprouts Fruit	Boiled rice Fish boiled with soy Pickles Boiled spinach Cooked spanish potato Food boiled down in soy souse Slight soup Boiled hijiki (brown algae)	Chicken bowl Miso soup Vinegared cucumber Sauted french bean Pickles Jelly	Boiled rice with fixing Roasted laver Tofu and ground meat dressed with miso Fried vegetable Egg roll Pickles Fruit

Total energy and nutrient contents of each meal set

	A	B	C	D
Energy (kcal)	1,512.0	1,796.7	1,776.2	1,654.4
Protein (g)	66.6	79.7	54.8	58.3
Calcium (mg)	466.2	406.4	317.1	330.0
Magnesium (mg)	220.5	217.4	184.3	267.0
Zinc (mg)	9.8	7.2	7.1	6.2
Dietary fiber (g)	14.4	15.3	14.9	16.9

Analytical methods. Duplicated meals were prepared and homogenized. The meals were dried at 110 ˚C for 48 h, wet-ashed with nitric acid, and diluted adequately with hydrochloric acid solution. The calcium contents of the meals were analyzed by the inductively coupled plasma atomic emission spectrometry (ICP-AES) method. The fecal samples were pretreated the same way as for the meals, and the concentrations of calcium were measured by atomic absorption spectrometry (Hitachi concentrations were measured by the OCPC method (Clinimate CA, Daiichi Pure Chemicals, Tokyo). Serum osteocalcin and plasma intact parathryroid hormone (PTH) levels were measured by immunoradiometric assay (BGP IRMA Mitsubishi, Mitsubishi Kagaku Latron, Tokyo; Allegro Intact PTH kit, Nichols Institute Diagnostics, San Clemente, CA). Calcitonin and 1,25-dihydroxyvitamin D_3 were analyzed by radioimmunoassay (Calcitonin RIA Mitsubishi, Mitsubishi Kagaku Latron, Tokyo; 1,25-dihydroxyvitamin D_3 RIA kit, TFB, Tokyo). Urinary deoxypyridinoline was measured by the CLIA method (ChemilumiACS-DPD, Sumitomo Pharmaceuticals, Osaka, Japan) and creatinine was measured by the Jaffe method (Creatinine-HR, Wako Pure Chemical Industries, Osaka, Japan). Deoxypyridinoline levels were adjusted for creatinine excretion, and were presented as nmol per mmol creatinine.

Calculation. Apparent calcium absorption and rate of absorption and calcium retention over the 4 d of the balance period were calculated as follows;

Apparent calcium absorption (mg/d) = daily calcium intake - daily calcium fecal excretion.

Absorption rate (%) = 100 × {(daily calcium intake - daily calcium fecal excretion)/daily calcium intake}.

Calcium retention (mg/d) = daily calcium intake - daily calcium fecal excretion - daily calcium urinary excretion.

Retention rate (%) = 100 × {(daily calcium intake - daily calcium fecal excretion - daily calcium urinary excretion)/daily calcium intake}.

Statistical analysis. Values of measured parameters are presented as the mean ± SEM. Comparisons between the experimental products were performed by paired-*t*-test (Statview version 5.0, SAS Institute, Cary, NC). Differences were considered significant at $P < 0.05$.

Results

Two subjects dropped out during the experiment due to common cold and diarrhea, confirmed not to have been caused by DFAIII ingestion by the subjects' records on the specified log sheet. Consequently, 12 subjects were finally analyzed in this study. The characteristics of the 12 subjects are shown in Table 2. The daily nutrient intakes of the subjects during 3 d are shown in Table 3. The average total calcium intake was 358 mg.

Table 2. Characteristics of the Subjects

Age (years)	23.0 ± 0.5
Height (cm)	169.6 ± 1.9
Weight (kg)	62.3 ± 2.8
BMI	21.5 ± 0.6

Values are presented as means ± SEM (n = 12).

Table 3. Subjects' Daily Nutrient Intake per D (mean of 3 d)

Energy (kcal)	1611 ± 57
Moisture (g)	786 ± 24
Protein (g)	50 ± 2
Lipids (g)	50 ± 2
Carbohydrates (g)	224 ± 10
Sodium (mg)	3048 ± 193
Potassium (mg)	1359 ± 40
Calcium (mg)	358 ± 28
Magnesium (mg)	147 ± 3
Phosphorus (mg)	674 ± 33
Ferrous (mg)	4.9 ± 0.2
Zinc (mg)	6.1 ± 0.3
Fiber (g)	8.7 ± 0.2

Calcium intake during the balance period was similar between the groups. Fecal excretion of calcium was 462 ± 26 mg/d in the DFAIII group, and 525 ± 29 mg/d in the control group. Therefore, the apparent calcium absorption (mg/d) and rate of absorption (%) were 43.8% and 39.1% higher respectively in the DFAIII group than in the control group. Urinary excretion of calcium was very similar between the control group and the DFAIII group. Consequently, the calcium retention (mg/d) and rate of retention (%) were much higher in the DFAIII group (Table 4).

Table 4. Absorption and Retention of Calcium during the 4-D Balance Period

Group	Intake (Ca mg/d)	Feces (Ca mg/d)	Urine (Ca mg/d)	Apparent absorption[a)] (mg/d)	Retention[b)] (mg/d)	Absorption rate[c)] (%)	Retention rate[d)] (%)
Control	677 ± 14	525 ± 29	138 ± 14	153 ± 22	15 ± 23	23 ± 4	2 ± 4
DFAIII	683 ± 16	462 ± 26	133 ± 14	220 ± 21*	87 ± 24*	32 ± 3*	13 ± 4*

Values are presented as means ± SEM (n = 12).

Significant differences between ingestion groups: *$P < 0.05$.

a) Apparent absorption = intake – feces

b) Retention = intake – feces – urine

c) Apparent absorption rate = {(intake – feces)/intake} × 100

d) Retention rate = {(intake – feces – urine)/intake} × 100

Concentrations of plasma intact parathyroid hormone (PTH), calcitonin 1,25-dihydroxyvitamin D_3, and calcium showed no differences between the two groups (Table 5). In comparisons between before and after the experimental period in each group, blood calcium increased and intact PTH decreased after ingestion of the test and the control food ($P < 0.05$). The concentration of 1,25-dihydroxyvitamin D_3 decreased only after the ingestion of food containing DFAIII ($P < 0.01$). Serum osteocalcin, a bone formation marker, increased in the DFAIII group ($P < 0.05$), but not in the control group (Fig. 2). The concentrations of urinary deoxypyridinoline, a bone resorption marker, and creatinine did not change throughout the experimental period (Table 6).

Table 5. Changes in Blood Parameters Related to Calcium Metabolism

	Control		DFAIII	
	Day 1	Day 14	Day 1	Day 14
Calcium (mg/dl)	9.48 ± 0.13	9.70 ± 0.07*	9.37 ± 0.12	9.68 ± 0.14*
1-25-di-hydroxyvitamin D_3 (pg/ml)	49.17 ± 3.06	49.25 ± 6.74	53.00 ± 3.26	42.67 ± 2.51**
PTH-INTACT (pg/ml)	42.08 ± 3.29	29.42 ± 2.55*	42.83 ± 2.44	28.00 ± 3.29*
Calcitonin (pg/ml)	27.92 ± 3.72	28.67 ± 3.06	25.86 ± 2.84	27.32 ± 2.88

Values are presented as means ± SEM (n = 12).

Significant differences *vs*. the value at day 1 in each group: *$P < 0.05$, ** $P < 0.01$.

Table 5. Changes in Urinary Creatinine and Deoxypridinoline Levels during the Experimental Period

	Day 1	Day 10–13 (balance period)
Creatinine (mg/dl)		
Control	1.14 ± 0.23	1.13 ± 0.06
DFAIII	0.81 ± 0.12	1.34 ± 0.10
Deoxypridinoline (nmol/mmol Cre)		
Control	6.06 ± 0.36	5.20 ± 0.12
DFAIII	7.05 ± 0.50	5.37 ± 0.16

Values are presented as means ± SEM (n = 12).

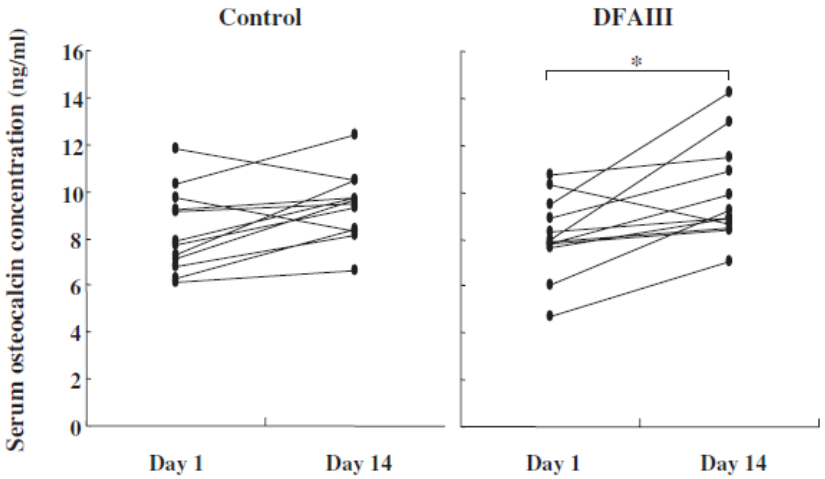

Fig. 2. Changes in Serum Osteocalcin Levels between Day 1 (pre-ingestion) and Day 14 (post-ingestion).

Values are presented as means ± SEM (n = 12). Significant differences *vs*. the value at day 1 (pre-ingestion) in each group: *$P < 0.05$.

Discussion

The aim of this study was to examine the effects of DFAIII on calcium absorption and retention using the balance method. We have reported that DFAIII promoted urinary calcium excretion in a human study.[25] This method offered only a suggestion, because urinary calcium clearance is not consistent with different renal functions and bone metabolism. The present study with a carefully designed balance method indicates that apparent calcium absorption and retention are increased by DFAIII in healthy men.

It was found that the daily nutrition intake of the subjects was less than complete. The calorie and protein intake of the subjects were relatively low compared with that in their age group of Japanese males. The subjects in this study consisted of students and part-time workers, and their lifestyle was thought to be irregular. In particular, the calcium intake of all subjects over 3 d was much lower than the average intake in Japanese males of ages 18–29, which is estimated to be 468 mg/d.[27] It can be predicted that the subjects in this study show low calcium intake in their daily life. Apparent calcium absorption in the balance period was increased 67 mg by DFAIII, but urinary calcium excretion was not affected. This led to increases in calcium retention in the DFAIII group. In this nutritional status, increased calcium supplied by promoting absorption by DFAIII can be efficiently retained and utilized in the body.

The mechanism by which DFAIII promotes calcium absorption is studied by fermentation in the large intestine, as with other nondigestible oligosaccharides. It has been reported that repeated ingestion of DFAIII changes intestinal microflora in humans.[28–30] Also, DFAIII promotes calcium absorption by acting directly on the paracellular transport pathway in the small intestinal epithelium.[11–14] Enhancement of calcium absorption by DFAIII has been reported in *in vivo* rat studies.[14] DFAIII increased calcium absorption in both the small and the large intestine, and the increase was greater than that of fructooligosaccharides, which promote calcium absorption only in the large intestine. Fructooligosaccharides and galactooligosaccharides have been found to improve absorption of calcium and magnesium, and the effects have been examined also in human

subjects.[31–36] However, some results of a stable isotope study failed to show improvement in calcium absorption due to fructooligosaccharides.[37] In the present study, we did not compare the effects of DFAIII with other nondigestible oligosaccharides. Further studies should clarify whether DFAIII has an advantage over other nondigestible saccharides in promoting calcium absorption.

Serum osteocalcin, a bone formation marker, increased after ingestion of DFAIII-containing food. This finding suggests that DFAIII might promote bone formation via increased calcium absorption. Osteocalcin in the control group also tended to increase at the end of the experimental period. Ingestion of 300 mg calcium in control food might also have affected calcium status and bone metabolism, because the subjects' daily calcium intakes were low. But we found much higher calcium retention in the DFAIII group. Since more than 99% of body calcium exists in bone, changes in the retention level of calcium can reflect the bone calcium level. The level of urinary deoxypyridinoline, a bone resorption marker, tended to be lower after ingestion (day 10–13) of both control and DFAIII foods than the level at day 1. These results suggest that increased calcium absorption by DFAIII preferentially promotes bone formation, more than it suppresses bone resorption. It has been found that feeding DFAIII to ovariectomized rats improves their bone strength and femoral mineral concentrations.[17,18] Long-term observation of changes in bone mass are required to evaluate the effects of DFAIII ingestion on bone metabolism in humans too.

Blood calcium significantly increased and plasma intact PTH decreased during both the control and the DFAIII test periods. The concentration of blood calcium and that of PTH are closely related. When blood calcium is low, PTH secretion is stimulated and bone resorption is enhanced. The daily calcium intake of the subjects was low at the beginning of the experiment, but increased to about 680 mg/day in the balance period (day 10–day 13). An improvement in nutritional status as to calcium in both groups might induce an increase in the blood calcium concentration and thereby, suppression of PTH secretion. In contrast, 1,25-dihydroxyvitamin D_3 decreased only in the DFAIII group subjects. The reason for this difference between the change in PTH and that in 1,25-dihydroxyvitamin D_3 is not known, but changes in PTH are perhaps much more sensitive to small changes in calcium

absorption than those in 1,25-dihydroxyvitamin D_3. Higher levels of 1,25-dihydroxyvitamin D_3 are known to have adverse effects on lipid metabolism.[38)]

In this study, we chose males as subjects so that the results would not be affected by menstruation-related changes in hormonal balance. Uenishi *et al*. have reported on the calcium requirement estimated by a balance study in young and elderly Japanese adults and mentioned gender difference in calcium balance according to body weight, sex hormones, and physical activity.[39,40)] Studies using female subjects are on the agenda to be examined in the future. The results of the present study indicate that DFAIII enhances calcium absorption and retention in human subjects. They also suggest that co-ingestion of calcium and DFAIII might make a contribution to efficacious bone formation.

References

1) National Institutes of Health, Osteoporosis prevention, diagnosis, and therapy. *NIH Consensus. Statement*, 17, 1–45 (2000).

2) Heaney, R. P., Recker, R. R., and Weaver, C. M., Absorbability of calcium sources: the limited role of solubility. *Calcif. Tissue Int*., 46, 300–304 (1990).

3) Uenishi, K., Ezawa, I., Kajimoto, M., and Tsuchiya, F., Calcium absorption from milk, fish (pond smelt, sardine) and vegetables (komatsuna-green, Jew's marrow, saltwort) in Japanese young women. *J. Jpn. Soc. Nutr. Food Sci*. (in Japanese), 51, 259–266 (1998).

4) Heaney, R. P., Weaver, C. M., and Fitzsimmons, M. L., Soybean phytate content: effect on calcium absorption. *Am. J. Clin. Nutr*., 53, 745–747 (1991).

5) Bullamore, J. R., Wilkinson, R., Gallagher, J. C., Nordin, B. E., and Marshall, D. H., Effects of age on calcium absorption. *Lancet*, 2, 535–537 (1970).

6) Hara, H., Suzuki, T., Kasai, T., Aoyama, Y., and Ohta, A., Ingestion of guar gum hydrolysate, a soluble fiber, increases calcium absorption in totally gastrectomized rats. *J. Nutr.*, 129, 39–45 (1999).

7) Shiga, K., Hara, H., Takahashi, T., Aoyama, Y., Furuta, H., and Maeda, H., Ingestion of water-soluble soybean fiber improves gastrectomy-induced calcium malabsorption and osteopenia in rats. *Nutrition*, 18, 636–642 (2002).

8) Mitamura, R., Hara, H., Aoyama, Y., Takahashi, T., and Furuta, H., Ingestion of water-soluble soybean fiber prevents osteopenia and hypercholesterolemia induced by ovariectomy in rats. *J. Agric. Food Chem.*, 51, 1085–1089 (2003).

9) Roberfroid, M. B., Concepts in functional foods: the case of inulin and oligofructose. *J. Nutr.*, 129, 1398S–1401S (1999).

10) Ohta, A., Ohtsuki, M., Baba, S., Adachi, T., Sakata, T., and Sakaguchi, E., Calcium and magnesium absorption from the colon and rectum are increased in rats fed fructooligosaccharides. *J. Nutr.*, 125, 2417–2424 (1995).

11) Mineo, H., Hara, H., Kikuchi, H., Sakurai, H., and Tomita, F., Various indigestible saccharides enhance net calcium transport from the epithelium of the small and large intestine of rats *in vitro*. *J. Nutr.*, 131, 3243–3246 (2001).

12) Mineo, H., Hara, H., Shigematsu, N., Okuhara, Y., and Tomita, F., Melibiose, difructose anhydride III and difructose anhydride IV enhance net calcium absorption in rat small and large intestinal epithelium by increasing the passage of tight junctions *in vitro*. *J. Nutr.*, 132, 3394–3399 (2002).

13) Mineo, H., Amano, M., Chiji, H., Shigematsu, N., Tomita, F., and Hara, H., Indigestible disaccharides open tight junctions and enhance net calcium, magnesium, and zinc absorption in isolated rat small and large intestinal epithelium. *Dig. Dis. Sci.*, 49, 122–132 (2004).

14) Suzuki, T., and Hara, H., Effects of difructose anhydride III on calcium absorption in small and large intestines of rats. *Biosci. Biotechnol. Biochem.*, 62, 837–841 (1998).

15) Mineo, H., Amano, M., Chiji, H., Shigematsu, N., Tomita, F., and Hara, H., Absorptive activity of calcium in the isolated cecal epithelium adaptively increased by 2 week's feeding of difructose anhydride III in rats. *Biosci. Biotechnol. Biochem.*, 67, 1847–1851 (2003).

16) Shiga, K., Hara, H., Okano, G., Ito, M., Minami, A., and Tomita, F., Ingestion of difructose anhydride III and voluntary running exercise independently increase femoral and tibial bone mineral density and bone strength with increasing calcium absorption in rats. *J. Nutr*., 133, 4207–4211 (2003).

17) Mitamura, R., Hara, H., Aoyama, Y., and Chiji, H., Supplemental feeding of difructose anhydride III restores calcium absorption impaired by ovariectomy in rats. *J. Nutr*., 132, 3387–3393 (2002).

18) Mitamura, R., and Hara, H., Prolonged feeding of difructose anhydride III increases strength and mineral concentrations of the femur in ovariectomized rats. Br. *J. Nutr*., 94, 268–274 (2005).

19) Li, H. Y., Hagiwara, H., Shu, W., Yokoyama, C., and Harada, N., Isolation and NMR studies of di-D-fructose anhydride III from lycoris radiate Herbert by supercritical extraction with carbon dioxide. *Carbohydr. Res.*, 299, 301–305 (1997).

20) Manley-Harris, M., and Richards, G. N., Di-D-fructose dianhydrides and related oligomers from thermal treatments of inulin and sucrose. *Carbohydr. Res.*, 287, 183– 202 (1996).

21) Ratsimba, V., Garcia-Fernandez, J. M., Defaye, J., Nigay, H., and Voilley, A., Qualitative and quantitative evaluation of mono- and disaccharides in D-fructose, D-glucose and sucrose caramels by gas-liquid chromatography–mass spectrometry. *J. Chromatogr. A*, 844, 283–293 (1999).

22) Defaye, J., and Garcia-Fernandez, J. M., The oligosaccharide components of caramels. *Zuckerrind.*, 120, 700–704 (1995).

23) Yokota, A., Hirayama, S., Enomoto, K., Miura, Y., Takao, S., and Tomita, F., Production of inulin fructotransferase (depolymerizing) by *Arthrobacter* sp. H65-7 and preparation of DFA III from inulin by the enzyme. *J. Ferment. Bioeng.*, 72, 258–261 (1991).

24) Yokota, A., Enomoto, K., and Tomita, F., Purification and properties of an inulin fructotransferase (depolymerizing) from *Arthrobacter* sp. H65-7. *J. Ferment. Bioeng.*, 72, 262–265 (1991).

25) Shigematsu, N., Okuhara, Y., Shiomi, T., Tomita, F., and Hara, H., Effect of difructose anhydride III on calcium absorption in humans. *Biosci. Biotechnol. Biochem.*, 68, 1011–1016 (2004).

26) Wang, D. H., Kogashiwa, M., Ohta, S., and Kira, S., Validity and reliability of a dietary assessment method: the application of a digital camera with a mobile phone card attachment. *J. Nutr. Sci. Vitaminol.* (Tokyo), 48, 498–504 (2002).

27) ‘‘The National Health and Nutrition Survey in Japan (in Japanese),’’ Ministry of Health, Labour and Welfare of Japan, *Daiichi Shuppan* (2003).

28) Tamura, A., Shiomi, T., Tamaki, N., Shigematsu, N., Tomita, F., and Hara, H., Comparative effect of repeated ingestion of difructose anhydride III and palatinose on the induction of gastrointestinal symptoms in humans. *Biosci. Biotechnol. Biochem.*, 68, 1882–1887 (2004).

29) Minamida, K., Sujaya, I. N., Tamura, A., Shigematsu, N., Sone, T., Yokota, A., Asano, K., Benno, Y., and Tomita, F., The effects of di-D-fructofuranose-1,2’:2,3’-dianhydride (DFA III) administration on human intestinal microbiota. *J. Biosci. Bioeng.*, 98, 244–250 (2004).

30) Minamida, K., Asakawa, C., Sujaya, I. N., Kaneko, M., Abe, A., Sone, T., Hara, H., Asano, K., and Tomita, F., Effects of long-term ingestion of difructose anhydride III (DFA III) on intestinal bacteria and bile acid metabolism in humans. *J. Biosci. Bioeng.*, 101, 149–156 (2006).

31) Griffin, I. J., Davila, P. M., and Abrams, S. A., Nondigestible oligosaccharides and calcium absorption in girls with adequate calcium intakes. *Br. J. Nutr.*, 87, S187–S191 (2002).

32) Coudray, C., Bellanger, J., Castiglia-Delavaud, C., Remesy, C., Vermorel, M., and Rayssignuier, Y., Effect of soluble or partly soluble dietary fibres supplementation on absorption and balance of calcium, magnesium, iron and zinc in healthy young men. *Eur. J. Clin. Nutr.*, 51, 375–380 (1997).

33) van den Heuvel, E. G., Schoterman, M. H., and Muijs, T., Transgalactooligosaccharides stimulate calcium absorption in postmenopausal women. *J. Nutr.*, 130, 2938–2942 (2000).

34) Morohashi, T., Sano, T., Ohta, A., and Yamada, S., True calcium absorption in the intestine is enhanced by fructooligosaccharide feeding in rats. *J. Nutr.*, 128, 1815–1818 (1998).

35) van den Heuvel, E. G., Muys, T., van Dokkum, W., and Schaafsma, G., Oligofructose stimulates calcium absorption in adolescents. *Am. J. Clin. Nutr.*, 69, 544–548 (1999).

36) Tahiri, M., Tressol, J. C., Arnaud, J., Bornet, F., Bouteloup-Demange, C., Feillet-Coudray, C., Ducros, V., Pepin, D., Brouns, F., Rayssiguier, A. M., and Coudray, C., Five-week intake of short-chain fructooligosaccharides increases intestinal absorption and status of magnesium in postmenopausal women. *J. Bone Miner. Res.*, 16, 2152–2160 (2001).

37) Tahiri, M., Tressol, J. C., Arnaud, J., Bornet, F. R., Bouteloup-Demange, C., Feillet-Coudray, C., Brandolini, M., Ducros, V., Pepin, D., Brouns, F., Roussel, A. M., Rayssiguier, Y., and Coudray, C., Effect of short-chain fructooligosaccharides on intestinal calcium absorption and calcium status in postmenopausal women: a stable-isotope study. *Am. J. Clin. Nutr.*, 77, 449–457 (2003).

38) Shi, H., Norman, A. W., Okamura, W. H., Sen, A., and Zemel, M. B., alpha,25-Dihydroxyvitamin D_3 modulates human adipocyte metabolism *via* nongenomic action. *FASEB J.*, 15, 2751–2753 (2001).

39) Uenishi, K., Ishida, H., Kamei, A., Shiraki, M., Ezawa, I., Goto, S., Fukuoka, H., Hosoi, T., and Orimo, H., Calcium requirement estimated by balance study in elderly Japanese people. *Osteoporos. Int.*, 12, 858–863 (2001), Erratum in: *Osteoporos. Int.*, 13, 184 (2002).

40) Nishimuta, M., Kodama, N., Morikuni, E., Yoshioka, Y. H., Takeyama, H., Yamada, H., Kitajima, H., and Suzuki, K., Balances of calcium, magnesium and phosphorus in Japanese young adults. *J. Nutr. Sci. Vitaminol.*, 50, 19–25 (2004).

Chapter 3

Dietary melibiose regulates Th cell response and enhances the induction of oral tolerance

It has been revealed that there are two different types of T helper (Th) cell-subsets, Th1 and Th2,[1] and that the cytokines derived from each Th cell regulate each other.[2,3] Th1-type cells produce interleukin (IL)-2 and interferon (IFN)-γ which activate macrophages and induce delayed-type hypersensitivity reactions. In contrast, Th2-type cells produce IL-4, IL-5 and IL-10 which induce IgE production. Thus, allergic disorders occur when the balance of Th1 and Th2 shifts toward Th2.

Lactic bacteria such as lactobacilli are well known to enhance the Th1-type immune system by IL-12 induction and suppress the Th2-type response.[4–6] Kalliomaki M *et al*.[7] have suggested that oral administration of lactobacilli was effective for preventing early atopic diseases in children. Bjorksten B. *et al*.[8] have reported that infants who developed allergy were less often colonized with bifidobacteria during the first year of life than healthy infants and that indigenous intestinal flora might play important roles in the development of and protection from allergy. On another front, prebiotics such as indigestible oligosaccharides are known to increase indigenous lactic bacteria, especially bifidobacteria in humans.[9–11] We have previously reported that dietary raffinose, an indigestible oligosaccharide, suppressed the Th2-type immune response against an oral antigen.[12] We also confirmed that disaccharide melibiose was produced when raffinose reacted with intestinal bacteria *in vitro*. This finding implied that the various physiological functions of raffinose might make their contribution in the form of melibiose.

Melibiose (6-o-α-D-galactopyranosyl-D-glucose) exists in natural plants such as cacao beans, and has also been found in processed soybeans. We have reported that the functions of melibiose included increasing lactic bacteria, especially bifidobacteria, and improving the stool condition in humans, as has been recognized with the other oligosaccharides.[13] We also performed a clinical test to investigate whether melibiose was useful for atopic dermatitis.[14] These results indicated that

melibiose had an effect on the immune system and could be useful for improving the symptoms of allergic diseases.

In the present study, we investigated the effects of dietary melibiose on immune responses, especially focusing on the Th cell response to an orally administrated protein antigen.

Materials and Methods

Animals. Female BALB/c mice were purchased from Clea Japan (Tokyo, Japan). OVA23-3 transgenic mice with a BALB/c genetic background expressing an OVA-specific I-A^d restricted αβ-T cell receptor were originally established by Sato *et al*.[15] Female transgenic mice were used for all the experiments. All experiments were performed in accordance with the guidelines for the care and use of laboratory animals of the University of Tokyo.

Culture medium. Lymphocytes were cultured in an RPMI-1640 medium (Nissui Phamaceutical Co., Tokyo, Japan) supplemented with heat-inactivated 10% fetal calf serum (Sigma, St. Louis, MO, USA), 100 U/ml of penicillin, 100 μg/ml of streptomycin, 5×10^{-5} M 2-mercaptoethanol and 2mM L-glutamine.

Antigen. OVA (5 × crystallized OVA; Seikagakukogyo, Tokyo, Japan) was used for adding to the culture medium and for needle feeding and immunization of BALB/c mice. As a food supplement, OVA (albumin from eggs, containing 500 g of OVA/kg) was obtained from Wako Pure Chemical Industries (Osaka, Japan).

Diets. The composition of the purified basal diet is shown in Table 1. The OVA diet was prepared by partly substituting OVA for casein in the basal diet. The melibiose diet and the melibiose-OVA diet were prepared by adding melibiose to the basal diets instead of cornstarch. The diets were

prepared in pellets and vacuum-sealed in plastic bags by Funabashi Farm (Chiba, Japan). Melibiose was used as melibiose monohydrate (Nippon Beet Sugar, Tokyo, Japan).

Table 1. Composition of the Diets (g/kg)

	basal diet	OVA diet	melibiose diet	melibiose-OVA diet
casein	200	120	200	120
cornstarch	481.7	481.7	431.7	431.7
α-starch	90	90	90	90
sucrose	50	50	50	50
cellulose	50	50	50	50
corn oil	60	60	60	60
mineral mixture	50	50	50	50
vitamin mixture	13	13	13	13
choline chloride	2.3	2.3	2.3	2.3
DL-methionine	3	3	3	3
egg albumin	—	80	—	80
melibiose	—	—	50	50

Effects of melibiose on cytokine production from transgenic mice fed with OVA. Female OVA23-3 mice at 4–5 weeks of age were maintained with the basal diet or the melibiose diet for 14 d. The basal diet was changed to the OVA diet, and the melibiose diet was changed to the melibiose-OVA diet. The mice were maintained for 0, 4, 7 and 10 d with each diet. Subsequently, the mice were sacrificed and their spleen cells and MLN cells were prepared from individual mice as a single cell suspension, and $CD4^+$ T cells were purified from MLN cells by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-CD4 beads.

Effects of the oral administration of melibiose on the T cell hyporesponsiveness (oral tolerance) induced in OVA-fed BALB/c mice. BALB/c mice (4 weeks of age) were fed with the basal diet or melibiose-containing diet for 14 d and assigned to three groups, the basal diet group, the basal diet plus OVA-fed group and the melibiose diet plus OVA-fed group. The mice in the basal diet plus OVA-fed and the melibiose diet plus OVA-fed groups were orally given 1 mg of OVA in saline by needle feeding, while the animals in the basal diet group were only given saline. Seven days after the oral administration of OVA/saline, all the BALB/c mice were subcutaneously (sc) immunized into the footpads and base of the tail with 50 mg of OVA in complete Freund's adjuvant (CFA, DIFCO,

Detroit, MI, USA). Mice were maintained for another 7 d and finally sacrificed. Their inguinal lymph nodes were dissected and separate cell suspensions were prepared.

Cell culture for cytokine production. Spleen cells and MLN cells (2.5×10^6/ml) from OVA 23-3 mice were cultured with OVA in 48-well plates (Costar, Cambridge, MA, USA) for the detection of IL-2 (for 36 h), and IL-4 and IFN-γ (for 60 h). In addition, MLN-$CD4^+$ T cells (5.0×10^5/ml) were cultured for cytokine production with OVA and with antigen-presenting cells (APC; 2.0×10^6/ml) over a suitable incubation time for each cytokine as already described. APC were prepared from the splenocytes of BALB/c mice maintained on a commercial diet, and the splenocyte cell suspensions were incubated with mitomycin C (50 μg/ml, 45 min at 37 °C; Sigma, St. Louis, MO, USA). Inguinal lymph node cells (2.5×10^6/ml) from BALB/c mice were cultured with OVA for 24 h to detect the levels of IL-2. The cells were also incubated for 65 h under the same conditions for IFN-γ detection.

Determination of the lymphocyte proliferative response. The lymphocyte proliferative response was determined by using a commercial 5-bromo-2'-deoxyuridine (BrdU) labeling and detection kit β (Roche Diagnostics, Mannheim, Germany). $CD4^+$ T cells of MLN (5.0×10^5/ml) were plated with APC (2×10^6/ml) into 96-well plates, and inguinal lymph node cells (2.5×10^6/ml) were also plated for cell culture. Each cell was then cultured with OVA for 38 h, and BrdU was added on hour 24. The detection of BrdU-incorporated cellular DNA was carried out according to the manufacturers' protocol. Briefly, the cultured cells were fixed with 0.5 M ethanol/HCl on to the plates, and a peroxidase-labeled anti-BrdU antibody was added. After adding the substrate, the absorbance at 405 nm was measured and therefore the amount of BrdU incorporated into the intracellular DNA could be evaluated.

ELISA for cytokines. The IL-2, IL-4, and IFN-γ levels were detected by sandwich ELISA. Rat anti-mouse IL-2, IL-4 and IFN-γ monoclonal antibodies were used as capture antibodies, with

biotinylated rat anti-mouse IL-2, IL-4 and IFN-γ respectively as the detection antibodies. All monoclonal antibodies were purchased from Pharmingen, San Diego, CA, USA.

Statistical analysis. Each result is presented as the mean value with SD. The differences in cytokine levels and cell proliferative activity between the experimental groups were analyzed with Student's *t*-test. Significant differences between values were measured by using Stat View (Ver 5.0). The level of significance was set at $P < 0.05$.

Results and Discussion

The aim of this study was to examine the effects of dietary melibiose on the immune response induced by an orally fed antigen. We demonstrate that dietary melibiose strongly suppressed the Th2 response and properly enhanced the oral tolerance induced by oral administration of the antigen OVA.

We evaluated the effects of melibiose on the T cell differentiation induced by the gradual oral administration of OVA to OVA 23-3 mice. OVA 23-3 mice express T-cell receptor αβ-chain genes derived from a clone of the OVA-specific I-A^d restricted $CD4^+$ T cell and are therefore useful for observing the T cell responses induced by the oral administration of antigen.[15] The mice have already been evaluated for the changes in cytokine production from their splenic T-cells after a long-term administration of OVA.[16] In that study, the IL-4 production increased and conversely IL-2 and IFN-γ production decreased after one week of ingesting OVA, showing the typical cytokine production patterns of Th2-type response.[16] However, it was confirmed that the splenic T cell cytokine production diminished after the OVA diet had been fed for more than 2 weeks.

We first observed the changes of cytokine production and T cell proliferative response at several stages of OVA sensitization. The mice in both the basal diet and melibiose diet group were further assigned to 4 groups (finally 8 groups, n = 3), and OVA was orally administrated for 0, 4, 7, or 10 d, respectively. Spleen cells and MLN cells from the mice were incubated with OVA, and each cytokine

level was measured. $CD4^+$ T cells were purified from MLN and incubated with APC for cytokine detection, because the IL-4 production level from whole MLN cells was negligible even in the presence of OVA *in vitro*. The cytokine production and proliferative response of the $CD4^+$ T cells from MLN are shown in Fig. 1. The cytokine production and proliferative response of $CD4^+$ T cells increased from the start of administration of OVA (day 0) to around day 7 and then started to decrease. These overall changes observed during the Th cell sensitization caused by OVA intake in the mice are consistent with the previous findings.[16] It was then noted that T cell proliferation in the melibiose diet group was significantly lower ($P < 0.05$) than that in the basal diet group and that the IL-4 production level tended to decrease ($P = 0.08$) on day 7. The IFN-γ production level also slightly decreased on day 7 compared with the basal diet group. Similar results were observed with spleen cells (data not shown).

We next performed an additional experiment in the same manner to observe the difference in cytokine production between the basal diet group (n = 10) and the melibiose diet group (n = 10) with the focus on day 7 (Fig. 2). As the representative results of two experiments, $CD4^+$ T cells of MLN from the melibiose diet group produced a significantly lower level of IL-4 than from the basal diet group on day 7 ($P < 0.05$). Spleen cells also showed a much more decreased IL-4 production level ($P < 0.01$) (data not shown). These results indicate that dietary melibiose strongly suppressed the Th2-type response induced by the oral administration of OVA. Furthermore, in both cells, IFN-γ production derived from the Th1 cell type tended to be lower in the melibiose diet group than in basal diet group. There was no significant difference in IL-2 production between the two groups.

Several researchers have reported on food components which had been expected to improve allergic disorders, and most of their effects depended on regulation of the Th1/Th2 balance.[4,5,17,18] Our present results show that dietary melibiose inhibited the Th2 response, but in addition to this, that the T cell proliferation was diminished and the Th1 response tended to decrease. Although there appeared to be a shift in the Th1/Th2 balance, the effects of melibiose on Tcell responses could not be explained solely by the regulation of the Th1/Th2 balance. It is also well known that the ingestion

of a food antigen can cause systemic hyporesponsiveness to the antigen, this being called oral tolerance.[19–24] Oral tolerance is one of the most important inherent immune-regulation systems and helps prevent a strong immune response to a dietary antigen that would be potentially harmful to the individual. It has been demonstrated in animals that such responses as specific antibody production, delayed-type hypersensitivity and the cytokine secretion of antigen-specific Th cells of the spleen or MLN were decreased by oral tolerance. We therefore explored the possibility that dietary melibiose also had some effects on the induction of oral tolerance through the administration of an antigen by using another evaluation model with BALB/c mice.

We performed a preliminary experiment to elucidate the dose and times of oral administration of OVA which were able to induce T cell hyporeactivity before examining the effects of melibiose on the T cell response with this experimental system. We were able to find the appropriate conditions for intragastric administration with 1 mg of OVA and then set up the additional experimental groups. The BALB/c mice were assigned to three groups, the basal diet group (n = 6), basal diet plus OVA-fed group (n = 8) and melibiose diet plus OVA-fed group (n = 8). After the administration schedule, the lymph node cell-proliferative response and cytokine production in each group were measured and compared (Fig. 3). The cell-proliferative response of the basal diet plus OVA-fed group tended to be lower than that of the basal diet group, the IL-2 response being significantly lower in the former than in the latter group. This shows that the Th cell responses normally induced when the mice are subcutaneously immunized with OVA were suppressed by a prior oral administration of OVA. Additionally, the response in the melibiose diet plus OVA-fed group was significantly lower than in the basal diet or basal diet plus OVA-fed group. Concerning IL-2 production, this showed almost the same pattern of that of the cell-proliferative activity, the differences observed between the groups actually being much clearer. As for IFN-γ production, no difference was apparent between the basal diet and basal diet plus OVA-fed group. However, the melibiose diet plus OVA-fed group demonstrated a lower IFN-γ level than that in the other groups. These results suggest that the phenomenon considered as the induction of oral tolerance was enhanced by dietary melibiose.

Although not shown by the data, we also performed a supplemental experiment to examine whether melibiose decreased the Th response without oral administration of OVA. The cell proliferative response and cytokine production induced by subcutaneous immunization of OVA were no different between the basal diet group and the melibiose diet group without prior OVA feeding.
This indicates that dietary melibiose only suppressed the Th response when the antigen was administrated orally.

At present, the mechanism by which dietary melibiose affects the Th cell response and oral tolerance remains unclear. However, many reports have stated that intestinal bacterial flora may affect immune responses; for instance, in germ-free mice which lack intestinal bacteria, IgA secretion is less than that of normal mice and oral tolerance is not induced.[25–27] Furthermore, it has also been reported that the kinds of T cell induced by invading bacteria or an antigen are different from those in normal mice. The fact that melibiose improved the intestinal flora as a prebiotic might lead to the hypothesis that dietary melibiose affects the intestinal immune system by modulating the intestinal flora and thereby regulating the systemic immune responses. There have also been some recent reports that oligosaccharides, which possess α-galactosyl binding activity, suppressed allergic airway eosinophilia and that the effect did not appear to be mediated by the intestinal microflora.[28,29] Although we have also examined in vitro whether melibiose had a direct influence on immune cells, no clear results have been obtained. In either case, further research to elucidate the mechanism of melibiose action is necessary.

In conclusion, we have shown that dietary melibiose significantly suppressed the Th2 response and enhanced oral tolerance induced by an orally fed antigen. These findings indicate the possibility that melibiose would be useful for preventing or improving the symptoms of allergic disease by regulating the Th cell response and that melibiose could well be a noteworthy food ingredient which could reinforce oral tolerance.

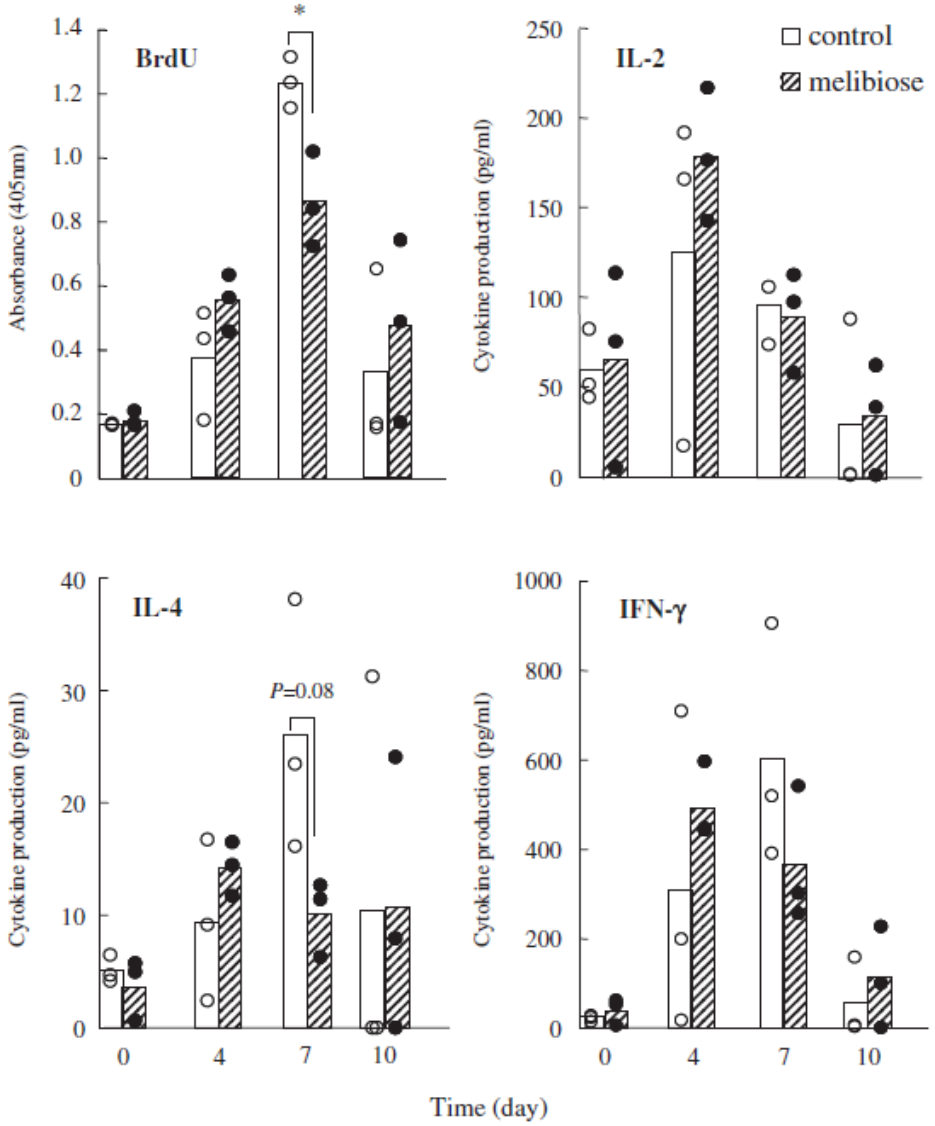

Fig. 1. Effect of Dietary Melibiose on the Cytokine Production and Proliferation Response of MLN CD4$^+$T Cells during Oral Sensitization with the OVA Antigen for Various Periods in TCR Transgenic Mice.

OVA23-3 mice (4 weeks old) were fed on the basal diet or the melibiose-containing diet for 14 d. Subsequently, each diet was changed to the respective OVA-containing diet, and the mice were maintained for another 0, 4, 7, or 10 d. CD4$^+$T cells of MLN were prepared and cultured with OVA (1.0 mg/ml) to detect the proliferative response and cytokine production. Columns indicate the mean values and circles represent the values for individual mice. ○ and :unfilled column, basal diet group; ●and hatched column, melibiose diet group.

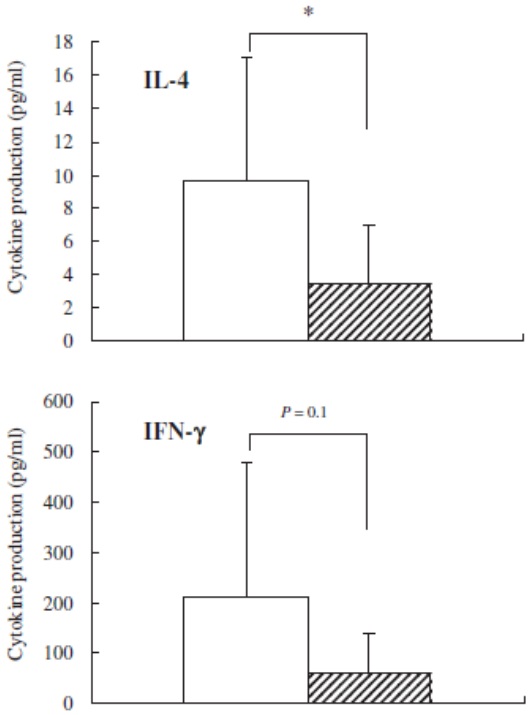

Fig. 2. Effect of Dietary Melibiose on Cytokine by MLN CD4$^+$T Cells of TCR Transgenic Mice Fed with OVA for 7d.

□ (unfilled column), basal diet group; ▨ (hatched column), melibiose diet group. Four-week old OVA23-3 mice were fed on the basal diet or melibiose-containing diet for 14 d. Subsequently, each diet was changed to the OVA-containing basal diet or OVAcontaining melibiose diet, respectively, and the mice were maintained for another 7 days. CD4$^+$ T cells from MLN were prepared and cultured with the antigen (OVA) for a cytokine assay. Each result is presented as the mean value plus or minus SD. The level of significance was set at *P < 0.05.and IL-2 and IFN-γ□ production. Each result is presented as the mean value plus or minus SD. The level of significance was set at *P < 0.05.

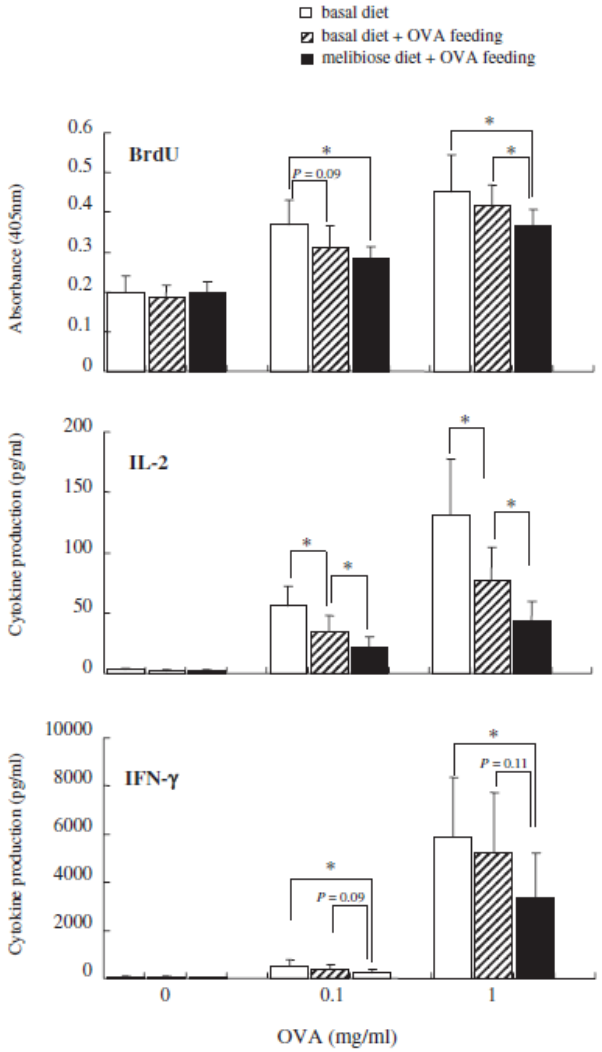

Fig. 3. Effect of Dietary Melibiose on the T Cell Hyperesponsiveness (Oral Tolerance) Induced by Oral Administration of OVA in BALB/c Mice.

Three-four-week-old mice were fed on the basal diet or the melibiose-containing diet for 14 d and then 1mg of OVA in saline or saline only was orally administrated by needle feeding. The mice were assigned to three groups: the basal diet group (unfilled column), the basal diet plus OVA-fed group (hatched column) and the melibiose diet plus OVA-fed group (filled column). Seven days after the oral administration of OVA/saline, all the mice were immunized subcutaneously (sc) with 50 mg of OVA in complete Freund's adjuvant and maintained for another 7 d. Their inguinal lymph nodes were dissected, and each cell suspension was prepared and cultured with antigen OVA for detecting the cell proliferative response and IL-2 and IFN-γ production. Each result is presented as the mean value plus or minus SD. The level of significance was set at *P < 0.05.

References

1) Mosmann, T. R., and Coffman, R. L., TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Ann. Rev. Immunol*., 7, 145–173 (1989).

2) Murphy, K. M., and Reiner, S. L., The lineage decisions of helper T cells. *Nat. Rev. Immunol*., 2, 933–944 (2002).

3) Fitch, F. W., McKisic, M. D., Lancki, D. W., and Gajewski, T. F., Differential regulation of murine T lymphocyte subsets. *Annu. Rev. Immunol*., 11, 29–48 (1993).

4) Shida, K., Makino, K., Morishita, A., Takamizawa, K., Hachimura, S., Ametani, A., Sato, T., Kumagai, Y., Habu, S., and Kaminogawa, S., Lactobacillus casei inhibits antigen-induced IgE secretion through regulation of cytokine production in murine splenocyte cultures. *Int. Arch. Allergy Immunol.*, 115, 278–287 (1998).

5) Shida, K., Takahashi, R., Iwadate, E., Takamizawa, K., Yasui, H., Sato, T., Habu, S., Hachimura, S., and Kaminogawa, S., Lactobacillus casei strain Shirota suppresses serum immunoglobulin E and immunoglobulin G1 responses and systemic anaphylaxis in a food allergy model. *Clin. Exp. Allergy,* 32, 563–570 (2002).

6) Murosaki, S., Yamamoto, Y., Ito, K., Inokuchi, T., Kusaka, H., Ikeda, H., and Yoshikai, Y., Heat-killed Lactobacillus plantarum L-137 suppresses naturally fed antigen-specific IgE production by stimulation of IL-12 production in mice. *J. Allergy Clin. Immunol.*, 102, 57–64 (1998).

7) Kalliomäki, M., Salminen, S., Arvilommi, H., Kero, P., Koskinen, P., and Isolauri, E., Probiotics in primary prevention of atopic disease: a randomised placebocontrolled trial. *Lancet*, 357, 1076–1079 (2001).

8) Björksten, B., Sepp, E., Julge, K., Voor, T., and Mikelsaar, M., Allergy development and the intestinal microflora during the first year of life. *J. Allergy Clin. Immunol., 108*, 516–520 (2001).

9) Van Loo, J., Cummings, J., Delzenne, N., Englyst, H., Franck, A., Hopkins, M., Kok, N., Macfarlane, G., Newton, D., Quigley, M., Roberfroid, M., van Vliet, T., and van den Heuvel, E., Functional food properties of non-digestible oligosaccharides: a consensus report from the ENDO project (DGXII AIRII-CT94-1095). *Br. J. Nutr.*, 81, 121–132 (1999).

10) Hidaka, H., Tashiro, Y., and Eida, T., Proliferation of Bifidobacteria by oligosaccharides and their useful effect on human health. *Bifidobacteria Microflora*, 10, 65–79 (1991).

11) Sako, T., Matsumoto, K., and Tanaka, R., Recent progress on research and applications of non-digestible galacto-oligosaccharides. *Int. Dairy J.*, 9, 69–80 (1999).

12) Nagura, T., Hachimura, S., Hashiguchi, M., Ueda, Y., Kanno, T., Kikuchi, H., Sayama, K., and Kaminogawa, S., Suppressive effect of dietary raffinose on T-helper 2 cell-mediated immunity. *Br. J. Nutr.*, 88, 421–426 (2002).

13) Nagura, T., Shimizu, Y., Sayama, K., and Benno, Y., Effect of melibiose on the fecal flora, with special reference to *Bifidobacterium* spp. and fecal properties in healthy volunteers. *Bifidus* (in Japanese), 9, 151–159 (1996).

14) Kaneko, I., Hayamizu, K., Tomita, K., Kikuchi, H., Nagura, T., Shigematsu, N., and Chiba, T., Pilot study of melibiose in patients with adolescent or adult-type atopic detmatitis. J. Appl. Glicosci. (in Japanese), 51, 123–128 (2004).

15) Sato, T., Sasahara, T., Nakamura, Y., Osaki, T., Hasegawa, T., Tadakuma, T., Arata, Y., Kumagai, Y., Katsuki, M., and Habu, S., Naive T cells can mediate delayed-type hypersensitivity response in T cell receptor transgenic mice. *Eur. J. Immunol.*, 24, 1512–1516 (1994).

16) Shida, K., Hachimura, S., Ametani, A., Ishimori, M., Ling, M., Hashiguchi, M., Ueda, Y., Sato, T., Kumagai, Y., Takamizawa, K., Habu, S., and Kaminogawa, S., Serum IgE response to orally ingested antigen: a novel IgE response model with allergen-specific T-cell receptor transgenic mice. *J. Allergy Clin. Immunol.*, 105, 788–795 (2000).

17) Nagafuchi, S., Hachimura, S., Totsuka, M., Takahashi, T., Goto, M., Yajima, T., Kuwata, T., Habu, S., and Kaminogawa, S., Dietary nucleotides can up-regulate antigen-specific Th1 immune responses and suppress antigen-specific IgE responses in mice. *Int. Arch. Allergy Immunol*., 122, 33–41 (2000).

18) Sudo, N., Aiba, Y., Takaki, A., Tanaka, K., Yu, X. N., Oyama, N., Koga, Y., and Kubo, C., Dietary nucleic acids promote a shift in Th1/Th2 balance toward Th1-dominant immunity. *Clin. Exp. Allergy*, 30, 979–987 (2000).

19) Faria, A. M., and Weiner, H. L., Oral tolerance. *Immunol. Rev.*, 206, 232–259 (2005).

20) Weiner, H. L., Oral tolerance. *Proc. Natl. Acad. Sci. USA*, 91, 10762–10765 (1994).

21) Marth, T., Strober, W., and Kelsall, B. L., High dose oral tolerance in ovalbumin TCR-transgenic mice: systemic neutralization of IL-12 augments TGF-β secretion and T cell apoptosis. *J. Immunol.*, 157, 2348–2357 (1996).

22) Yoshida, T., Hachimura, S., and Kaminogawa, S., The oral administration of low-dose antigen induces activation followed by tolerization, while high-dose antigen induces tolerance without activation. *Clin. Immunol. Immunopathol*., 82, 207–215 (1997).

23) Asai, K., Hachimura, S., Kimura, M., Toraya, T., Yamashita, M., Nakayama, T., and Kaminogawa, S., T cell hyporesponsiveness induced by oral administration of ovalbumin is associated with impaired NFAT nuclear translocation and $p27^{kip1}$ degradation. *J. Immunol*., 169, 4723–4731 (2002).

24) Ise, W., Nakamura, K., Shimizu, N., Goto, H., Fujimoto, H., Kaminogawa, S., and Hachimura, S., Orally tolerized T cells can form conjugates with APCs but are defective in immunological synapse formation. *J. Immunol*., 175, 829–838 (2005).

25) Sudo, N., Sawamura, S., Tanaka, K., Aiba, Y., Kubo, C., and Koga, Y., The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J. Immunol*., 159, 1739–1745 (1997).

26) Wannemuehler, M. J., Michalek, S. M., Jirillo, E., Williamson, S. I., Hirasawa, M., and McGhee, J. R., LPS regulation of the immune response: bacteroides endotoxin induces mitogenic, polyclonal,

and antibody responses in classical LPS responsive but not C3H/He. J mice. *J. Immunol*., 133, 299–305 (1984).

27) Umesaki, Y., Setoyama, H., Matsumoto, S., Imaoka, A., and Itoh, K., Differential roles of segmented filamentous bacteria and clostridia in development of the intestinal immune system. *Infect. Immun*., 67, 3504–3511 (1999).

28) Sonoyama, K., Watanabe, H., Watanabe, J., Yamaguchi, N., Yamashita, A., Hashimoto, H., Kishino, E., Fujita, K., Okada, M., Mori, S., Kitahata, S., and Kawabata, J., Allergic airway eosinophilia is suppressed in ovalbuminsensitized Brown Norway rats fed raffinose and alphalinked galactooligosaccharide. *J. Nutr.*, 135, 538–543 (2005).

29) Watanabe, H., Sonoyama, K., Watanabe, J., Yamaguchi, N., Kikuchi, H., Nagura, T., Aritsuka, T., Fukumoto, K., and Kasai, T., Reduction of allergic airway eosinophilia by dietary raffinose in Brown Norway rats. *Br. J. Nutr.*, 92, 247–255 (2004).

Chapter 4

Ninety-day dietary toxicity study of apple polyphenol extracts in Crl: CD (SD) rats

1. Introduction

Apples contain several types of polyphenols. These include procyanidin, epicatechin, catechin, p-coumaroyl quinic acid, chlorogenic acid, rutin, and phloridzin; the main components of polyphenols are oligomeric procyanidins.[1-6] Applephenon® (AP) is a polyphenol extract produced from the juice of unripe apples using column chromatography. It consists mainly of procyanidins, which are composed of epicatechin and catechin.[7]

AP has been reported to have various physiological effects. These include anti-allergic effects,[8-12] lowering effects on serum cholesterol,[13] inhibitory effects on post-prandial levels of triglycerides [14] and anti-obesity effects [15-16] *in vivo*. Antioxidant effects, [17-18] antitumor effects,[19-22] inhibitory effects on pancreatic lipase activity [23] and micellar solubilization *in vitro* [24] have also been suggested. Furthermore, recent research has indicated that procyanidins from apples have sir-2.1-dependent anti-aging effects on *Caenorhabditis elegans*.[25] Focusing on these effects, AP has been used widely as a dietary supplement or as an antioxidant food additive for various processed foods in Japan. It was generally recognized as safe (GRAS) in 2004 in accordance with regulations set by the Food and Drug Administration (FDA) in the USA. We have reported on the safety of AP using the Ames test, acute-oral toxicity test, and 90-day subchronic-toxicity test.[26] In the latter, AP was administered every day to Crl: CD (SD) rats at 0, 500, 1000 or 2000 mg/kg body weight (BW) by oral gavage. However, the oral toxicity of other food materials and food additives were examined primarily by the 90-day oral feeding test, in which the method of administration is close to the manner of daily intake of food materials in meals. [27-33] Therefore, in the present study, we undertook another 90-day subchronic test to examine further the safety of dietary AP in feeds at 0%, 1.25%, 2.5% and 5.0%.

2. Materials and methods

This study was carried out in accordance with Guidelines for Designation of Food Additives and for Revision of Standards for Use of Food Additives of Japan (1996).

2.1. Test materials and diet

AP (Asahi Food and Healthcare Co. Ltd., Tokyo, Japan) was used. It was prepared from unripe apple juices (*Malus pumila* cv. Fuji) by solid phase extraction with SEPABEADS® SP-70 (Mitsubishi Chemical Corporation, Tokyo, Japan). [6] The composition of AP is shown in Table 1.

Test diets were prepared by mixing 0%, 1.25%, 2.5%, or 5.0% AP to the basal diet (CRF-1: Oriental Yeast Co., Ltd., Tokyo, Japan) and all of these were gammasterilized. The uniformity and stability of AP in each test diet was confirmed using a published method.[34] These diets were kept in a refrigerator at 4.2–6.5 ˚C throughout the test period.

Table 1. Content of polyphenol in AP. [a]

Content	(%)
Procyanidins	
dimer	11.1 [b]
trimer	12.3 [b]
tetramer	8.7 [b]
pentamer	5.9 [b]
hexamer	4.9 [b]
over heptamer	20.9 [b]
Flavan-3-ols	
(+)-catechin	2.0 [c]
(–)-epicatechin	10.5 [c]
Chalcones	
phloridzin	1.9 [c]
phloretin-2'-xyloglucoside	4.6 [c]
Phenolcarboxylic Acids	
chlorogenic acid	8.2 [c]
p-coumaroyl quinic acid	2.6 [c]

[a] Each component was identified by NMR and spectral analysis (procyanidins and phloretin-2'-xyloglucoside) or by comparison of chromatograms with authentic standards ((+)-catechin, (–)-epicatechin, phloridzin, and chlorogenic acid).
[b] Analyzed by normal phase-HPLC.
[C] Analyzed by reversed phase-HPLC.

2.2. Animals

Eighty-eight (44 of each sex) 4-week-old Crl: CD (SD) rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The general condition of all animals was checked daily for 12 days and gains in BW noted. They underwent ophthalmological examinations in quarantine as well as an acclimatization period before study initiation. Forty healthy rats of each sex were finally selected for the study. Five of the remaining animals were used for the assessment of viral infections and the other three rats were excluded as subjects and killed.

3. Experimental

The present study was carried out by Mitsubishi Chemical Medience Corporation (Tokyo, Japan) in compliance with Good Laboratory Practice (GLP) and in accordance with the guidelines for animal studies of Mitsubishi Chemical Medience Corporation.

Male and female rats (age, 6 weeks; 40 rats of each sex) were maintained individually in stainless-steel cages at 23.1–24.3 ˚C, relative humidity (55.1–66.8%) and 12-h lighting (07:00–19:00). They were divided into four groups and given feed containing the test material at 0% (control), 1.25%, 2.5%, or 5.0% for 90 days. Drinking water was also provided to the animals ad libitum. The initiation day and week of the administration was each set as ‘‘day 1’’ and ‘‘week 1’’, respectively.

The viability of the animals was observed twice daily. BW and food consumption were measured every 7 days from day 1 to day 85, and day 90. Food efficiency (food consumption/weight gain (%)) and the mean intake of test articles (mg/kg BW/day) were calculated from BW and food consumption, respectively. Water consumption was also measured for 24 h from day 87 to day 88. Furthermore, an ophthalmological examination was done on 5 selected animals at the end of the administration period (day 89).

Urinalyses were conducted at week 13 (days 87–88). Animals were put into the metabolism cages individually. Fresh urine samples were collected for a fixed period (07:41–09:22). Subsequently, 24-h urine samples were collected under the fasting (but freewater drinking) condition. These

collected samples were stored at -80 ˚C. Fresh urine samples were used for the measurement of pH, protein, glucose, ketone body, bilirubin, occult blood, and urobilinogen using urinary test strips (Pretest 8aII; Wako Pure Chemical Industries Ltd., Osaka, Japan). The volume (mL/24 h) and color of urine was measured using urine samples collected over 24 h. In addition, samples were used for the measurement of osmotic pressure (osm/kg) by an OSMOMAT 030-D-RS Osmometer (Gonotec, Berlin, Germany) as well as levels of sodium, potassium and chloride (mEq/24 h) using a PVA-aIII system (Analytical Instruments Co., Ltd., Tokyo, Japan). Diets and drinking water were given in the usual way after the collection of fresh urine.

After the 90-day administration period, rats were fasted overnight (17–22 h). Blood samples were collected from the abdominal aorta under anesthesia (pentobarbital sodium, 30 mg/kg bw, i.p.). They were mixed with ethylenediamine tetra-acetic acid (EDTA) and used for hematological tests by employing an ADVIA 120 system (Bayer Diagnostics Manufacturing Ltd., Newbury, UK). The parameters were white blood cells (WBCs), red blood cell (RBCs), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), sickle cell count, platelet count and WBC differential count. The other blood samples were mixed with 3.8% (w/v) of sodium citrate and centrifuged at 1870g for 15 min to collect the plasma. In addition, serum was collected by centrifugal separation (1870g for 10 min at room temperature). The plasma was used for the blood coagulation test to obtain the prothrombin time (PT) and activated partial thromboplastin time (APTT) (Sysmex CA-5000; Sysmex Co., Kobe, Japan). Serum samples were assessed using an autoanalyzer (7170; Hitachi, Ltd., Tokyo, Japan) for the following biochemical parameters: total protein (T. protein), total bilirubin (T. bilirubin), aspartate aminotransferase (AST), alanine aminotransferase (ALT), c-glutamyl transpeptidase (GTP), alkaline phosphatase (ALP), total cholesterol (T. cholesterol), triglycerides, phospholipids, glucose, blood urea nitrogen (BUN), creatinine, inorganic phosphorus (IP), calcium, and magnesium. Measurements for creatine phosphate kinase (CPK) and lactate dehydrogenase (LDH) were made using plasma. Differential serum protein and the ratio of albumin/globulin (A/G) were analyzed using an AES320 system

(Olympus Co., Tokyo, Japan). Blood levels of sodium, potassium, and chloride were analyzed by a PVA-aIII system (Analytical Instruments Co., Ltd.).

Subsequently, rats were killed and necropsy findings recorded. The brain, pituitary gland, submandibular glands, thyroid glands, heart, lungs, thymus, liver, spleen, kidneys, adrenal glands, seminal vesicles, prostate gland, testes, ovaries, and the uterus were removed and their weights (absolute weights) recorded. Relative weights (calculated by dividing the organ weight by the final BW on day 91) were also documented.

Samples of the following organs and tissues were removed and fixed in phosphate-buffered 10% formalin for histopathological evaluation: tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, submandibular glands, sublingual glands, parotid glands, liver, pancreas, nasal cavity, trachea, lungs, thymus, submandibular lymph node, mesenteric lymph node, spleen, bone-marrow, heart, aorta, kidney, urinary bladder, prostate, seminal vesicle, ovary, oviduct, uterus, vagina, mammary gland, pituitary gland, thyroid glands, parathyroid gland, adrenal glands, cerebrum, cerebellum, medulla oblongata, spinal cord, sciatic nerve, Harderian gland, Zymbal's gland, musculus biceps femoris, sternum, femur and integument. Eyes and optic nerves were fixed in Davidson's solution. The testes and epididymis were fixed in Bouin's solution. Histopathological tests were carried out in animals in the control group and 5.0% group of each sex. In addition, the submandibular glands in the 2.5% groups and parotid glands in the 1.25% and 2.5% groups of sexes were also investigated.

3.1. Statistical analyses

The following parameters were analyzed using the MiTOX-PPL system (Mitsui Zosen Systems Research Inc., Chiba, Japan): BW, food consumption, food efficiency, water consumption, as well as the respective parameters of urinalysis, hematology and blood chemistry. Each mean value and the standard deviation were calculated and the homogeneity of variance analyzed. Differences between the control group and each test group were evaluated using Dunnett's test or Steel's test. $P < 0.05$

was considered significant.

4. Results

4.1. Clinical observations

Death or clinical signs were not observed in any group throughout the study.

4.2. Bw

In the 5.0% group of males, BW was significantly decreased compared with the control group on day 22 and day 64 ($P < 0.05$), and was also shown from day 36 to day 90 in the 5.0% group of females (day 36, 43, 50 and 64; $P < 0.05$, day 57, 71, 78, 85 and 90; $P < 0.01$) (Table 2). Conversely, there were no significant differences between the other test diet groups (1.25%, 2.5%) and the control.

Table 2. Body weights of male and female fed each diet for 90 days (*N* = 10/sex).

Diet group		Day	1	8	15	22	29	36	43	50	57	64	71	78	85	90
Male	0%	Mean	202.4	267.0	320.6	365.1	411.1	447.2	472.9	498.7	521.7	542.5	570.4	589.1	605.5	602.6
		SD	11.3	17.2	20.7	20.1	26.9	32.3	35.6	37.4	39.3	41.9	49.4	52.9	56.1	54.8
	1.25%	Mean	200.3	260.9	315.3	357.4	398.8	433.4	459.3	482.7	501.8	524.4	554.3	574.9	590.9	592.2
		SD	10.9	14.2	14.3	19.1	21.6	26.4	29.7	30.7	33.6	36.6	48.6	52.8	56.2	60.0
	2.5%	Mean	203.5	261.6	314.1	350.9	394.5	428.9	455.0	479.3	499.4	522.6	547.2	564.1	580.8	579.4
		SD	10.9	16.2	17.1	21.4	22.2	26.6	30.6	29.4	32.9	40.6	42.4	45.1	46.3	45.6
	5.0%	Mean	204.2	256.4	306.0	341.6	387.1	424.0	449.6	475.0	488.5	494.8 *	531.7	551.2	561.5	563.8
		SD	9.8	12.0	11.2	16.3	15.5	17.1	17.0	14.1	18.4	19.2	22.4	22.2	23.0	25.6
Female	0%	Mean	162.9	187.6	212.5	234.1	250.0	264.5	277.9	286.4	297.7	302.9	316.6	322.0	329.5	332.1
		SD	7.4	12.0	14.9	17.1	18.8	20.2	24.3	24.8	26.3	28.9	34.3	35.1	35.2	38.3
	1.25%	Mean	162.1	186.2	210.0	227.8	243.5	255.8	265.9	274.8	285.8	292.5	300.0	305.4	312.3	311.3
		SD	8.3	10.8	15.6	15.6	17.5	17.7	17.7	20.5	21.2	23.0	25.8	23.8	23.1	25.4
	2.5%	Mean	162.7	186.3	206.3	222.3	239.2	250.9	259.9	265.4	277.6	283.5	290.8	293.2	300.2	298.3
		SD	7.5	10.2	13.6	15.9	21.9	24.6	24.6	30.5	32.2	32.1	38.8	39.8	42.8	46.1
	5.0%	Mean	161.3	183.4	203.0	219.1	232.8	242.5 *	250.2 *	255.6 *	263.0 **	270.6 *	274.0 **	277.2 **	284.2 **	281.5 **
		SD	7.8	10.7	9.8	12.1	12.6	12.9	12.9	11.6	11.9	11.9	11.5	14.0	12.7	13.4

Data are mean ± SD values.

*Significant different from the controls as $P < 0.05$.

**Significant different from the controls as $P < 0.01$.

4.3. Consumption of food and water/food efficiency

There were no changes in food consumption between the groups (Table 3). In the 5.0% groups, a significant decrease in food efficiency was shown on day 8, 22, 57, 64 and 85 in males (day 22; $P < 0.05$, day 8, 57, 64 and 85; $P < 0.01$), and on day 15, 22, 43, 71 and 90 in females (day 15, 22, 43 and 90; $P < 0.05$, day 71; $P < 0.01$). These changes were sporadic and not accompanied by poor nutritional status. Decreases in food efficiency were also observed on day 8 in the 2.5% groups of males and on day 15 and 22 in those of females ($P < 0.05$), but were transient at the beginning of administration only (data not shown). Water consumption was not significantly different in any group compared with the control group (data not shown).

Table 3. Food consumptions of male and female rats fed each diet for 90 days (N = 10/sex).

Diet group		Day	8	15	22	29	36	43	50	57	64	71	78	85	90
Male	0%	Mean	23.8	25.5	26.3	27.4	27.5	27.4	27.7	27.0	26.7	28.7	28.5	27.7	23.7
		SD	2.1	1.7	2.1	2.3	2.3	2.8	2.8	2.7	2.6	3.5	3.6	3.4	6.3
	1.25%	Mean	23.3	25.5	25.7	26.7	26.9	26.8	26.8	26.6	27.3	29.1	28.8	28.1	25.4
		SD	1.5	1.8	2.1	2.3	2.8	2.5	2.4	2.7	3.0	4.1	4.1	3.4	2.9
	2.5%	Mean	23.6	25.8	25.2	26.9	27.3	27.2	27.1	26.1	27.3	28.6	28.0	27.7	25.5
		SD	1.8	1.5	1.2	1.6	1.6	1.9	2.0	1.7	2.2	2.1	1.7	1.4	1.2
	5.0%	Mean	23.0	26.4	26.3	28.1	28.7	28.8	28.7	27.4	26.6	29.8	29.5	28.8	26.5
		SD	1.6	1.4	1.6	1.2	0.9	1.0	0.9	0.9	0.6	1.2	1.4	1.6	1.9
Female	0%	Mean	16.4	16.8	17.4	17.4	18.4	18.5	18.1	18.2	17.8	18.3	18.2	17.6	17.4
		SD	1.4	1.4	1.7	1.8	2.3	1.7	1.8	2.0	2.5	3.0	2.5	2.0	1.9
	1.25%	Mean	16.4	17.0	17.5	17.3	17.4	17.6	17.6	18.0	17.4	17.7	18.2	18.0	16.8
		SD	0.8	1.2	1.0	1.2	1.2	0.8	1.0	0.7	1.0	1.2	1.2	2.0	1.7
	2.5%	Mean	16.8	17.3	17.8	18.1	18.1	18.2	18.3	18.5	18.0	18.0	18.5	18.5	18.7
		SD	1.2	1.7	1.6	2.3	2.1	2.4	2.3	2.5	2.2	2.9	2.6	3.1	2.5
	5.0%	Mean	16.5	17.6	18.0	17.9	18.0	17.8	18.0	17.7	17.7	17.7	18.0	18.2	17.8
		SD	1.1	1.1	1.1	0.9	1.1	1.0	0.7	1.2	1.3	1.3	1.1	0.9	2.7

Data are mean ± SD values.

4.4. Article intake

The mean daily intake of AP in the 1.25%, 2.5%, and 5.0% groups was 752, 1521, and 3214 mg/kg bw in males and 836, 1769, and 3621 mg/kg BW in females, respectively.

4.5. Ophthalmological examination

Ophthalmological examinations undertaken on 5 selected rats did not show abnormalities.

4.6. Urinalyses

Although slight differences were observed in urinary osmotic pressures of the 1.25% and 5.0% groups compared with the control, there were no AP-related changes in any other parameters (data not shown).

4.7. Hematology/serum biochemistry

Dietary AP did not adversely affect hematology and serum biochemistry in any group. In the 5.0% AP group of males, PT was prolonged ($P < 0.05$ *versus* control). In the 5.0% AP group of females, the APTT was also prolonged ($P < 0.05$), and the numbers of eosinophils, neutrophils, basophils and monocytes were decreased compared with the control ($P < 0.05$, $P < 0.01$) (Tables 4 and 5). These changes were slight and comparable with that in the control group if referring to individual data. Therefore, these were not considered to be toxic changes. The results of serum biochemistry are shown in Tables 6 and 7. In the 5.0% group of males, the level of chloride in serum was lower than that in the control group ($P < 0.05$). However, this change was comparable with that observed in the control group. Additionally, serum albumin and the ratio of A/G in that group were significantly increased compared with that in the control group ($P < 0.05$). Significant decreases in the b-globulin level in the 2.5% group of females and the albumin level in the 1.25% group of females were also observed. However, there were not dose-related.

Table 4. Hematology findings of male rats fed each diet for 90 days (*N* = 10/sex).

Diet group		0 %	1.25 %	2.5 %	5.0 %	
Leukocytes	(10^3/μL)	9.16 ± 1.37	9.84 ± 2.23	9.25 ± 1.71	9.93 ± 2.41	
Erythrocytes	(10^4/μL)	884 ± 45	879 ± 29	889 ± 42	860 ± 23	
Hemoglobin	(g/dL)	15.4 ± 1.1	15.4 ± 0.7	15.3 ± 0.3	15.3 ± 0.5	
Hematocrit	(%)	44.5 ± 2.5	44.1 ± 1.6	44.0 ± 1.1	43.5 ± 1.1	
MCV	(fL)	50.4 ± 2.6	50.2 ± 2.1	49.5 ± 2.2	50.7 ± 1.7	
MCH	(pg)	17.5 ± 1.1	17.5 ± 0.9	17.2 ± 0.7	17.8 ± 0.7	
MCHC	(g/dL)	34.6 ± 0.5	34.8 ± 0.5	34.7 ± 0.4	35.1 ± 0.5	
Reticulocyte	(10^4/μL)	13.7 ± 5.5	13.6 ± 1.7	13.0 ± 2.5	12.6 ± 2.0	
Platelets	(10^4/μL)	89.0 ± 20.4	98.8 ± 9.8	93.1 ± 6.2	91.6 ± 13.1	
PT	(s)	13.8 ± 1.0	13.7 ± 0.9	14.1 ± 1.0	15.2 ± 1.6	*
APTT	(s)	21.4 ± 1.2	21.4 ± 1.5	21.6 ± 1.5	21.5 ± 2.0	
Differential count of WBC						
Eosinophils	(10^2/μL)	1.5 ± 0.7	1.6 ± 0.6	1.7 ± 0.5	1.4 ± 0.5	
Neutrophils	(10^2/μL)	12.7 ± 4.2	13.9 ± 4.7	17.2 ± 6.0	16.4 ± 4.9	
Lymphocytes	(10^2/μL)	74.7 ± 11.5	79.4 ± 18.5	70.3 ± 13.2	77.9 ± 21.9	
Basophils	(10^2/μL)	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	
Monocytes	(10^2/μL)	1.9 ± 0.6	2.4 ± 0.7	2.2 ± 0.5	2.3 ± 0.4	
Large instained cells	(10^2/μL)	0.7 ± 0.2	0.9 ± 0.3	0.9 ± 0.7	1.1 ± 0.6	

Data are mean ± SD values.

*Significant different from the controls as $P < 0.05$.

Table 5. Hematology findings of female rats fed each diet for 90 days (*N* = 10/sex).

Diet group		0 %	1.25 %	2.5 %	5.0 %	
Leukocytes	(10^3/μL)	6.53 ± 1.34	6.84 ± 1.77	6.04 ± 1.64	5.00 ± 1.13	
Erythrocytes	(10^4/μL)	787 ± 46	801 ± 32	789 ± 21	787 ± 36	
Hemoglobin	(g/dL)	14.4 ± 0.7	14.4 ± 0.5	14.2 ± 0.4	14.1 ± 0.8	
Hematocrit	(%)	40.6 ± 1.8	41.0 ± 1.6	40.8 ± 1.3	40.4 ± 2.0	
MCV	(fL)	51.7 ± 1.2	51.2 ± 1.1	51.7 ± 1.8	51.3 ± 1.2	
MCH	(pg)	18.3 ± 0.5	18.0 ± 0.4	18.0 ± 0.6	18.0 ± 0.6	
MCHC	(g/dL)	35.4 ± 0.3	35.1 ± 0.5	34.8 ± 0.5	35.0 ± 0.7	
Reticulocyte	(10^4/μL)	12.4 ± 2.7	12.0 ± 2.1	11.6 ± 3.8	11.4 ± 2.8	
Platelets	(10^4/μL)	98.7 ± 4.7	100.1 ± 8.7	98.5 ± 10.5	102.4 ± 11.2	
PT	(s)	12.4 ± 0.5	12.4 ± 0.7	12.3 ± 0.4	12.0 ± 0.5	
APTT	(s)	17.4 ± 1.1	17.7 ± 0.6	17.6 ± 1.2	18.6 ± 0.8	*
Differential count of WBC						
Eosinophils	(10^2/μL)	1.0 ± 0.3	1.2 ± 0.5	0.9 ± 0.4	0.7 ± 0.1	*
Neutrophils	(10^2/μL)	10.9 ± 3.1	10.5 ± 5.5	8.8 ± 3.4	7.1 ± 2.1	**
Lymphocytes	(10^2/μL)	50.9 ± 13.3	54.2 ± 12.8	48.8 ± 15.2	40.7 ± 10.7	
Basophils	(10^2/μL)	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	*
Monocytes	(10^2/μL)	1.7 ± 0.8	1.7 ± 0.6	1.3 ± 0.5	1.0 ± 0.3	*
Large intestine cells	(10^2/μL)	0.7 ± 0.3	0.7 ± 0.3	0.5 ± 0.2	0.5 ± 0.2	

Data are mean ± SD values.

*Significant different from the controls as $P < 0.05$.

**Significant different from the controls as $P < 0.01$.

Table 6. Biochemical findings of male rats fed each diet for 90 days (*N* = 10/sex).

Diet group		0 %	1.25 %	2.5 %	5.0 %	
T. Protein	(g/dL)	5.6 ± 0.2	5.8 ± 0.2	5.6 ± 0.3	5.6 ± 0.2	
Albumin	(g/dL)	2.61 ± 0.12	2.65 ± 0.14	2.69 ± 0.12	2.76 ± 0.11	*
A/G ratio		0.87 ± 0.07	0.85 ± 0.08	0.92 ± 0.09	0.96 ± 0.07	*
α1-Globulin	(g/dL)	1.28 ± 0.16	1.35 ± 0.13	1.26 ± 0.21	1.21 ± 0.10	
α2-Globulin	(g/dL)	0.39 ± 0.05	0.41 ± 0.03	0.40 ± 0.04	0.41 ± 0.04	
β-Globulin	(g/dL)	0.96 ± 0.08	0.97 ± 0.08	0.92 ± 0.05	0.90 ± 0.06	
γ-Globulin	(g/dL)	0.39 ± 0.07	0.39 ± 0.05	0.36 ± 0.05	0.36 ± 0.04	
T. Bilirubin	(mg/dL)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
AST	(IU/L)	144 ± 168	86 ± 10	84 ± 9	86 ± 7	
ALT	(IU/L)	35 ± 43	21 ± 3	23 ± 4	25 ± 3	
γ-GTP	(IU/L)	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.1	
ALP	(IU/L)	172 ± 52	157 ± 24	160 ± 23	162 ± 20	
LDH	(IU/L)	106 ± 42	88 ± 35	92 ± 28	82 ± 28	
CPK	(IU/L)	97 ± 32	112 ± 67	131 ± 52	122 ± 55	
T. Cholesterol	(mg/dL)	73 ± 14	76 ± 5	78 ± 17	78 ± 7	
Triglycerides	(mg/dL)	56 ± 23	68 ± 26	59 ± 28	69 ± 29	
Phospholipids	(mg/dL)	114 ± 19	126 ± 10	125 ± 24	126 ± 12	
Glucose	(mg/dL)	120 ± 11	122 ± 14	124 ± 15	118 ± 12	
BUN	(mg/dL)	15.3 ± 1.9	13.9 ± 2.5	13.8 ± 1.3	14.4 ± 1.8	
Creatinine	(mg/dL)	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	
IP	(mg/dL)	6.1 ± 0.5	6.4 ± 0.5	6.3 ± 0.3	6.2 ± 0.4	
Ca	(mg/dL)	9.9 ± 0.4	10.1 ± 0.3	10.0 ± 0.3	10.2 ± 0.2	
Mg	(mg/dL)	2.1 ± 0.1	2.0 ± 0.1	2.0 ± 0.2	2.0 ± 0.1	
Na	(mEq/L)	145.4 ± 0.7	145.1 ± 0.5	145.0 ± 0.8	145.0 ± 0.7	
K	(mEq/L)	4.39 ± 0.25	4.42 ± 0.09	4.43 ± 0.11	4.40 ± 0.17	
Cl	(mEq/L)	104.9 ± 0.8	103.8 ± 1.0	104.3 ± 1.6	103.6 ± 0.9	*

Data are mean ± SD values.

*Significant different from the controls as $P < 0.05$.

Table 7. Biochemical findings of female rats fed each diet for 90 days (*N* = 10/sex).

Diet group		0 %	1.25 %	2.5 %	5.0 %	
T. Protein	(g/dL)	6.4 ± 0.5	5.9 ± 0.3	6.0 ± 0.4	6.1 ± 0.5	
Albumin	(g/dL)	3.59 ± 0.29	3.22 ± 0.34	* 3.45 ± 0.25	3.44 ± 0.37	
A/G ratio		1.30 ± 0.19	1.19 ± 0.17	1.36 ± 0.09	1.31 ± 0.09	
α1-Globulin	(g/dL)	1.19 ± 0.31	1.10 ± 0.08	1.08 ± 0.11	1.07 ± 0.12	
α2-Globulin	(g/dL)	0.36 ± 0.04	0.36 ± 0.03	0.35 ± 0.01	0.33 ± 0.03	
β-Globulin	(g/dL)	0.82 ± 0.07	0.85 ± 0.07	0.75 ± 0.04	* 0.78 ± 0.03	
γ-Globulin	(g/dL)	0.43 ± 0.07	0.42 ± 0.10	0.37 ± 0.04	0.46 ± 0.07	
T. Bilirubin	(mg/dL)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	
AST	(IU/L)	127 ± 125	81 ± 16	80 ± 18	107 ± 38	
ALT	(IU/L)	52 ± 73	23 ± 10	26 ± 9	28 ± 9	
γ-GTP	(IU/L)	0.0 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	
ALP	(IU/L)	67 ± 19	70 ± 8	56 ± 7	86 ± 48	
LDH	(IU/L)	131 ± 95	80 ± 29	75 ± 26	90 ± 33	
CPK	(IU/L)	112 ± 77	98 ± 78	84 ± 48	86 ± 35	
T. Cholesterol	(mg/dL)	103 ± 38	85 ± 15	82 ± 13	87 ± 16	
Triglycerides	(mg/dL)	86 ± 110	34 ± 9	37 ± 16	32 ± 19	
Phospholipids	(mg/dL)	197 ± 68	157 ± 22	157 ± 22	165 ± 32	
Glucose	(mg/dL)	123 ± 10	120 ± 11	116 ± 11	115 ± 8	
BUN	(mg/dL)	14.8 ± 1.7	14.7 ± 1.7	15.4 ± 2.7	15.7 ± 2.3	
Creatinine	(mg/dL)	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	
IP	(mg/dL)	5.1 ± 0.8	5.6 ± 0.7	5.4 ± 0.7	5.2 ± 0.7	
Ca	(mg/dL)	10.7 ± 0.5	10.3 ± 0.3	10.6 ± 0.1	10.4 ± 0.4	
Mg	(mg/dL)	2.1 ± 0.1	2.1 ± 0.0	2.1 ± 0.1	2.0 ± 0.1	
Na	(mEq/L)	144.4 ± 0.8	144.0 ± 0.7	144.2 ± 1.5	144.1 ± 1.0	
K	(mEq/L)	4.01 ± 0.38	4.00 ± 0.17	4.08 ± 0.20	3.95 ± 0.21	
Cl	(mEq/L)	105.3 ± 1.8	105.8 ± 1.2	106.4 ± 1.6	106.3 ± 1.8	

Data are mean ± SD values.

*Significant different from the controls as $P < 0.05$.

4.8. Organ weights

In the 5.0% groups, absolute heart weight was decreased ($P < 0.05$) and the relative brain weight was increased ($P < 0.01$) in both sexes. The absolute weight of the kidneys was also decreased ($P < 0.05$) and the relative weight of the submandibular glands was increased in the 5.0% AP group of females ($P < 0.01$). The relative weights of lungs and ovaries were increased in the 2.5% ($P < 0.05$ for both) and 5.0% ($P < 0.01$) AP groups of females. In addition, the absolute pituitary weight in the 1.25% and 2.5% female groups was decreased but not in the 5.0% group ($P < 0.05$) (Tables 8 and 9).

4.9. Necropsy observations/histopathology

Necropsy changes were not observed (data not shown). Histopathological changes are shown in Table 10. Slight hypertrophy in the acinar cells of parotid glands in the 2.5% and 5.0% groups as well as those of the submandibular glands in the 5.0% groups were observed in males and females, and were related to AP treatment. In contrast, in the 5.0% group, one female rat showed lymphocytic infiltration in the Harderian gland, and two females showed infiltration of mononuclear cells in the biceps femoris muscle. However, these changes were not thought to be due to the test materials because all of these grade signs were determined to be ‘‘slight’’ and are also frequently observed in healthy rats. The changes appearing in other organs and tissues were observed with almost equal frequency in the test groups and the control group, or only in the control group.

Table 8. Absolute and relative organ weights of male rats fed each diet for 90 days (*N* = 10/sex).

Diet group	0 %	1.25 %	2.5 %	5.0 %	
Final body weight (g)	575.1 ± 51.4	563.5 ± 55.3	551.2 ± 44.0	532.1 ± 24.5	
Absolute organ weights (g)					
Brain	2.20 ± 0.07	2.21 ± 0.07	2.18 ± 0.10	2.26 ± 0.09	
Pituitary	0.015 ± 0.001	0.015 ± 0.002	0.014 ± 0.002	0.014 ± 0.001	
Submandibullar grands	0.89 ± 0.11	0.89 ± 0.15	0.86 ± 0.12	0.91 ± 0.09	
Thyroids	0.025 ± 0.005	0.028 ± 0.006	0.025 ± 0.003	0.024 ± 0.005	
Heart	1.90 ± 0.25	1.73 ± 0.22	1.70 ± 0.22	1.64 ± 0.09	*
Lungs	1.77 ± 0.29	1.70 ± 0.16	1.72 ± 0.20	1.69 ± 0.11	
Thymus	0.36 ± 0.09	0.33 ± 0.11	0.33 ± 0.08	0.31 ± 0.06	
Liver	14.99 ± 2.11	14.27 ± 2.17	13.83 ± 1.36	13.62 ± 1.11	
Spleen	0.93 ± 0.14	0.86 ± 0.19	0.84 ± 0.11	0.92 ± 0.14	
Kidneys	3.69 ± 0.40	3.51 ± 0.27	3.44 ± 0.34	3.40 ± 0.25	
Adrenals	0.065 ± 0.010	0.064 ± 0.010	0.062 ± 0.009	0.064 ± 0.007	
Seminal vesicle	2.66 ± 0.31	2.68 ± 0.34	2.67 ± 0.33	2.51 ± 0.20	
Prostate	0.90 ± 0.20	0.96 ± 0.15	0.83 ± 0.15	0.89 ± 0.13	
Testes	3.71 ± 0.35	3.67 ± 0.29	3.51 ± 0.21	3.66 ± 0.29	
Relative organ weights (g/100 g bw)					
Brain	0.38 ± 0.03	0.39 ± 0.03	0.39 ± 0.02	0.43 ± 0.03	**
Pituitary	0.003 ± 0.000	0.003 ± 0.000	0.003 ± 0.000	0.003 ± 0.000	
Submandibullar grands	0.16 ± 0.02	0.16 ± 0.01	0.16 ± 0.02	0.17 ± 0.01	
Thyroids	0.004 ± 0.001	0.005 ± 0.001	0.005 ± 0.001	0.005 ± 0.001	
Heart	0.33 ± 0.03	0.31 ± 0.02	0.31 ± 0.04	0.31 ± 0.02	
Lungs	0.31 ± 0.05	0.30 ± 0.02	0.31 ± 0.02	0.32 ± 0.02	
Thymus	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.01	
Liver	2.60 ± 0.25	2.52 ± 0.20	2.51 ± 0.19	2.56 ± 0.19	
Spleen	0.16 ± 0.02	0.15 ± 0.02	0.15 ± 0.02	0.17 ± 0.03	
Kidneys	0.64 ± 0.04	0.63 ± 0.05	0.62 ± 0.06	0.64 ± 0.03	
Adrenals	0.011 ± 0.001	0.011 ± 0.001	0.011 ± 0.002	0.012 ± 0.001	
Seminal vesicle	0.46 ± 0.05	0.48 ± 0.05	0.49 ± 0.05	0.47 ± 0.03	
Prostate	0.16 ± 0.03	0.17 ± 0.02	0.15 ± 0.02	0.17 ± 0.02	
Testes	0.65 ± 0.04	0.66 ± 0.07	0.64 ± 0.04	0.69 ± 0.05	

Data are mean ± SD values.

*Significant different from the controls as $P < 0.05$.

**Significant different from the controls as $P < 0.01$.

Table 9. Absolute and relative organ weights of female rats fed each diet for 90 days (*N* = 10/sex).

Diet group	0 %	1.25 %	2.5 %	5.0 %
Final body weight (g)	312.8 ± 34.6	292.9 ± 23.5	282.2 ± 42.4	266.2 ± 12.5 **
Absolute organ weights (g)				
Brain	1.96 ± 0.09	1.98 ± 0.08	1.93 ± 0.09	2.03 ± 0.08
Pituitary	0.020 ± 0.005	0.016 ± 0.002 *	0.016 ± 0.003 *	0.019 ± 0.004
Submandibullar grands	0.53 ± 0.05	0.52 ± 0.06	0.53 ± 0.07	0.56 ± 0.07
Thyroids	0.019 ± 0.003	0.018 ± 0.002	0.019 ± 0.004	0.017 ± 0.002
Heart	1.04 ± 0.11	0.97 ± 0.06	0.94 ± 0.12	0.91 ± 0.05 *
Lungs	1.25 ± 0.12	1.22 ± 0.12	1.19 ± 0.13	1.15 ± 0.05
Thymus	0.30 ± 0.06	0.27 ± 0.08	0.28 ± 0.05	0.26 ± 0.04
Liver	8.20 ± 1.19	7.51 ± 0.53	7.39 ± 1.28	7.16 ± 0.63
Spleen	0.59 ± 0.11	0.53 ± 0.08	0.52 ± 0.13	0.52 ± 0.05
Kidneys	1.99 ± 0.24	1.90 ± 0.12	1.82 ± 0.12	1.78 ± 0.14 *
Adrenals	0.069 ± 0.009	0.069 ± 0.008	0.069 ± 0.012	0.066 ± 0.007
Ovaries	0.065 ± 0.017	0.078 ± 0.016	0.077 ± 0.019	0.076 ± 0.014
Uterus	0.65 ± 0.17	0.60 ± 0.12	0.60 ± 0.13	0.57 ± 0.10
Relative organ weights (g/100 g bw)				
Brain	0.63 ± 0.06	0.68 ± 0.05	0.69 ± 0.10	0.76 ± 0.02 **
Pituitary	0.007 ± 0.002	0.006 ± 0.001	0.006 ± 0.001	0.007 ± 0.001
Submandibullar grands	0.17 ± 0.02	0.18 ± 0.02	0.19 ± 0.01	0.21 ± 0.02 **
Thyroids	0.006 ± 0.001	0.006 ± 0.001	0.007 ± 0.001	0.006 ± 0.001
Heart	0.33 ± 0.02	0.33 ± 0.03	0.34 ± 0.02	0.34 ± 0.01
Lungs	0.40 ± 0.03	0.42 ± 0.02	0.42 ± 0.03 *	0.43 ± 0.01 **
Thymus	0.09 ± 0.01	0.09 ± 0.02	0.10 ± 0.02	0.10 ± 0.02
Liver	2.62 ± 0.24	2.57 ± 0.10	2.61 ± 0.11	2.69 ± 0.19
Spleen	0.19 ± 0.04	0.18 ± 0.02	0.18 ± 0.01	0.20 ± 0.02
Kidneys	0.64 ± 0.07	0.65 ± 0.05	0.65 ± 0.08	0.67 ± 0.06
Adrenals	0.022 ± 0.003	0.024 ± 0.003	0.024 ± 0.003	0.025 ± 0.002
Ovaries	0.021 ± 0.006	0.027 ± 0.006	0.027 ± 0.004 *	0.028 ± 0.005 **
Uterus	0.21 ± 0.06	0.21 ± 0.04	0.21 ± 0.04	0.21 ± 0.03

Data are mean ± SD values.

*Significant different from the controls as $P < 0.05$.

**Significant different from the controls as $P < 0.01$.

Table 10. Histopathological findings of male and female rats fed each diet for 90 days (*N* = 10/sex).

	Male				Female			
Diet group	0 %	1.25 %	2.5 %	5.0 %	0 %	1.25 %	2.5 %	5.0 %
Organ and findings [a]								
Stomach								
Erosion, glandular stomach	1	-	-	0	0	-	-	0
Submandibullar glands								
Hypertrophy, acinus	0	-	0	2	0	-	0	3
Parotid glands								
Hypertrophy, acinus	0	0	5	10	0	0	3	9
Cellular infiltration, mononuclear cell	2	1	1	2	1	1	0	0
Mineralization, duct	0	0	0	0	1	0	1	0
Liver								
Necrosis, hepatocyte, centrilobular	1	-	-	0	0	-	-	0
Proliferaion, bile, duct	1	-	-	0	0	-	-	0
Cellular infiltration, mononuclear cell	8	-	-	8	6	-	-	8
Pancreas								
Cellular infiltration,lymphocyte	2	-	-	2	3	-	-	2
Lung								
Metaplasia, osseous	2	-	-	0	0	-	-	0
Accumulation, foam cell, alveolus	4	-	-	6	3	-	-	2
Mineralization, artery	2	-	-	7	3	-	-	4
Heart								
Cellular infiltration, mononuclear cell	2	-	-	0	1	-	-	0
Kidney								
Tubule, basophilic	5	-	-	0	1	-	-	0
Cast, proteinaceous	2	-	-	0	1	-	-	0
Cyst	2	-	-	0	0	-	-	0
Cellular infiltration, lymphocyte	3	-	-	2	3	-	-	1
Mineralization, medulla	3	-	-	1	4	-	-	1
Prostate								
Cellular infiltration, lymphocyte	5	-	-	4				
Ovary								
Cyst					2	-	-	1
Pituitary								
Cyst, anterior lobe	1	-	-	1	1	-	-	0
Tyroid								
Remnant, ultimobranchial body	0	-	-	0	3	-	-	1
Eye								
Dysplasia, retina	1	-	-	0	0	-	-	0
Harderian gland								
Cellular infiltration, lymphocyte	0	-	-	0	0	-	-	1
M. biceps femoris								
Cellular infiltration, mononuclear cell	0	-	-	0	0	-	-	2

[a] Number of animals with tissues examined histopathologically. All findings were recognized as “slight”.

5. Discussion

In the present study, the 90-day dietary toxicity of AP in feed was investigated in Crl: CD (SD) rats.

A decrease in BW was shown in the 5.0% groups of both sexes but was not accompanied by any other clinical deterioration. This finding was suggested to be related to the physiological function of AP and procyanidin contained in AP, such as the inhibitory effect on pancreatic lipase activity [23] and an antiobesity effect.[15-16] It was considered that the significant decrease in food efficiency was also guided by the decrease in body weight because food consumption between the groups did not change. The observed changes in organ weights, absolute heart weight, and relative brain weight in males and females, the absolute weight of the kidneys and the relative weight of the submandibular glands in the 5.0% female group, and the relative weights of lungs and ovaries in the 2.5% and 5.0% female groups, were also considered to be related to the significant decrease (or its tendency) of BW by AP administration. Therefore, these changes were not recognized as toxic changes.

Increases in serum albumin level and the ratio of A/G in the 5.0% group of males were also considered to be affected by AP administration. However, they were not accompanied by abnormal changes in clinical signs. In general, the increases in serum albumin level were not reported as toxicological findings, whereas the decreases in this parameter would have been recognized as toxic changes, thereby suggesting hepatic disorders and nutritional deficiency. Thus, toxicological changes were not suggested.

In histopathological analyses, slight hypertrophy of the acinar cells in the parotid glands and submandibular glands were observed in the 2.5% and 5.0% groups. There were no changes in organ weights and cytotoxicity or injury (e.g., degeneration, necrosis, inflammation) in the salivary glands. These findings were not observed in the single-dose and 90-day subchronic test by gavage administration of AP.[26] This suggested that the hypertrophy of acinar cells observed in the present study was not a systemic action but a localized, responsive reaction in the oral cavity induced by consecutive high-dose administration of an AP-contained diet.

The components of AP are similar to those of other polyphenolic extracts such as green tea catechins (GTC) and grape seed extract (GSE). The safety of GTC and GSE has been demonstrated by employing the 90-day oral feeding study using rats.[27-30] In those studies, histopathological changes were also not observed in the salivary glands. However, the administration dose of these agents in feed was not exactly the same as in the current study (maximum dose of GSE, 2.0%; of GTC, 5.0%). The main polyphenols in GTC are flavan-3-ols such as epigallocatechin gallate and catechins,[35] and those in GSE are proanthocyanidins.[36-38] Hence, GTC and GSE have astringency derived from procyanidins as well as AP. In contrast, AP contains mainly procyanidins as well as phenolcarboxylic acid derivatives, flavonols and other flavonoids. In particular, phenolcarboxylic acid derivatives such as chlorogenic acid characteristically present in a greater concentration in AP than in products of GTC and GSE. Hence, AP has a peculiar sour taste and lower pH of than that of GSE and GTC. Actually, the pH of 1.0% aqueous AP was 2.91, and that of GSE and GTC was 4.01 and 4.05, respectively. With this information, it was speculated that the slight hypertrophy of acinar cells in the salivary glands observed in the present study might have been affected not by the astringency of procyanidins but by the lower pH derived from derivatives of phenolcarboxylic acid.

The similar findings observed in the salivary glands have been reported in the 90-day oral feeding toxicity study of other food ingredients given to rats. In the study of L-aspartic acid (L-Asp), acinar-cell hypertrophy of the submandibular and parotid glands was observed in P2.5% doses of L-Asp in male and females.[39] The authors could not decide on the toxicological significance of these findings induced by L-Asp, but mentioned the involvement of a sour taste of diets containing 2.5% and 5.0% of L-Asp. In a study of *N*-acetyl-L-aspartic acid (NAA), acinar-cell hypertrophy of the salivary glands was also observed in a NAA-containing diet group.[40] The authors suggested that the decreased pH of the test diet resulted in stimulation of the salivary glands.

It has been reported that enlargement of the salivary glands in experimental animals might be associated with several agents, and can be classified according to the postulated mechanism involving a: (i) neural mechanism; (ii) endocrine mechanism; and (iii) miscellaneous (alternation).[41]

Burdock et al.[42] reported that 35-day oral feeding of the enzymes involved in the improved yield of 50-nucleotides (RP-1® and DN-50000®) in rats induced an increase in the weight of the submandibular glands and acinar-cell hypertrophy, but this was not observed in administration of a similar dose by oral gavage. The authors indicated that these effects were similar to those of pancreatin. Pancreatin stimulates taste receptors in the oral cavity, initiating a neural reflex arc and causing increased autonomic stimulation of the submandibular glands, thereby resulting in hypertrophy.[43] Furthermore, Wells and Peronace [44] hypothesized that hypertrophic effects of a bulk diet are the result of an intense reflex stimulation of the glands because of excessive intake of food. The functional stimulus is the need to provide additional saliva to moisten food for mastication and deglutition. Thus, saliva production induced by a lower pH derived from dietary ingredients could also be considered to be a neural reflex that is controlled by a parasympathetic effect. Conversely, the increased prevalence of acinar-cell hypertrophy of the submandibular and sublingual glands accompanied by increases in their absolute weights were observed in a 13-week oral toxicity study of guarana extract in feed to rats were considered to be due to caffeine contained in the extract.[45] Furthermore, these findings using caffeine were also observed in a single-dose toxicity test by oral gavage.[46] The mechanism of the enlargement of the salivary glands by caffeine was suggested to be an adaptive response to a sympathomimetic effect. Taken together, we concluded that the slight hypertrophy of acinar cells of the submandibular and parotid glands observed in the present study were not toxicological but instead a transient physiological adaptive response to an oral stimulus by consecutive lowering of pH of the AP-contained diet.

Furthermore, we made a comparative investigation of these results versus that of the previous 90-day subchronic-toxicity study by oral gavage of AP.[26] In our previous study, death and toxic changes were not observed at a maximum dose of 2000 mg/kg/day, but several necropsy and histopathological findings were considered to be related to AP administration. Briefly, red-brown discolorations and histological accumulation of pigmented phagocytic macrophages were observed in the digestive tract of AP-treated groups. Similar effects in the digestive tract were not observed

even at a maximum dose of 5.0% AP in the current study. The daily consumption of AP in the 5.0% group was estimated to be 3214 mg/kg/day in males and 3621 mg/kg/day in females. These values were much higher that of the previous oral gavage study (2000 mg/kg/day). Additionally, it was confirmed that the red-brown granules stained positive in the histochemical periodic acid-Schiff stain and were not derived from hemorrhage or bile by other staining methods. These findings suggested that the previous AP-related findings in the digestive tract were not toxicological effects but adaptive responses affected by consecutive administration of high concentrations of AP by the oral gavage method.

6. Conclusion

These data suggest that AP did not show toxicological effects in the 90-day oral feeding test and can be safely used as a food material. The no observed adverse effect level (NOAEL) of AP was considered to be 5.0% in males and females (male: 3214 mg/kg/day; female: 3621 mg/kg/day).

References

1) Spanos, G.A., Ronald, E.W., Heatherbell, D.A., Influence of processing and storage on the phenolic composition of apple juice. *J. Agri. Food Chem.*, 38, 1572–1579 (1990).

2) Lister, C.E., Lancaster, J.E., Sutton, K.H., Walker, J.R.L., Developmental changes in the concentration and composition of flavonoids in skin of a red and a green apple cultivar. *J. Sci. Food Agri.*, 64, 155–161 (1994).

3) Ohnishi-Kameyama, M., Yanagida, A., Kanda, T., Nagata, T., Identification of catechin oligomer from apple (*Malus pumila* cv. Fuji) in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and fast-atom bombardment mass spectrometry. *Rapid Commun. Mass Spectrom.*, 11, 31–36 (1997).

4) Suárez, B., Picinelli, A., Moreno, J., Mangas, J.J., Changes in phenolic composition of apple juices by HPLC with direct injection. *J. Sci. Food Agri.*, 78, 461–465 (1998).

5) Mangas, J.J., Rodríguez, R., Suárez, B., Picinelli, A., Dapena, E., Study of the phenolic profile of cider apple cultivars at maturity by multivariate techniques. *J. Agri. Food Chem.*, 47, 4046–4052 (1999).

6) Shoji, T., Mutsuga, M., Nakamura, T., Kanda, T., Akiyama, H., Goda, Y., Isolation and structural elucidation of some procyanidins from apple by lowtemperature NMR. *J. Agri. Food Chem.*, 51, 3806–3813 (2003).

7) Shoji, T., Masumoto, S., Moriichi, N., Kanda, T., Ohtake, Y., Apple (Malus pumila) procyanidins fractionated according to the degree of polymerization using normal-phase chromatography and characterized by HPLC-ESI/MS and MALDI-TOF/MS. *J. Chromatogr.*, 1102, 206–213 (2006).

8) Kanda, T., Yanagida, A., Tanabe, M., Akiyama, H., Goda, Y., Toyoda, M., Teshima, R., Saito, Y., Inhibitory effects of apple polyphenol on induced histamine release from RBL-2H3 cells and rat mast cells. *Biosci. Biotechnol. Biochem.*, 62, 1284–1289 (1998).

9) Akiyama, H., Sakushima, J., Teshima, R., Toyoda, M., Taniuchi, S., Kojima, T., Kobayashi, Y., Kanda, T., Yanagida, A., Antiallergic effect of apple polyphenols on the allergic model mouse. *Biol. Pharm. Bull.*, 23, 1370–1373 (2000).

10) Akiyama, H., Sato, Y., Watanabe, T., Nagaoka, M.H., Yoshioka, Y., Shoji, T., Kanda, T., Yamada, K., Totsuka, M., Teshima, R., Sawada, J., Goda, Y., Maitani, T., Dietary unripe apple polyphenol inhibits the development of food allergies in murine models. *FEBS Lett.*, 579, 4485–4491 (2005).

11) Nakano, N., Nishiyama, C., Tokura, T., Nagasako-Akazome, Y., Ohtake, Y., Okumura, K., Ogawa, H., Procyanidin C1 from apple extracts inhibits Fc epsilon RImediated mast cell activation. *Int. Arch. Allergy Immunol.*, 147, 213–221 (2008).

12) Enomoto, T., Nagasako-Akazome, Y., Kanda, T., Ikeda, M., Dake, Y., Clinical effects of apple polyphenols on persistent allergic rhinitis: a randomized double-blind placebo-controlled parallel arm study. *J. Invest. Allergol. Clin. Immunol.*, 16, 283–289 (2006).

13) Nagasako-Akazome, Y., Kanda, T., Ikeda, M., Shimasaki, H., Serum cholesterollowering effect of apple polyphenols in healthy subjects. *J. Oleo Sci.*, 54, 143–151 (2005).

14) Nagasako-Akazome, Y., Kanda, T., Sugiyama, H., Ohtake, Y., Shimasaki, H., Efficacy of suppression on postprandial serum triglyceride elevation with apple polyphenols. *Jpn. J. Food Chem.,* 14, 82–86 (2007).

15) Osada, K., Funayama, M., Fuchi, S., Effects of dietary procyanidins and tea polyphenols on adipose tissue mass and fatty acid metabolism in rats on a high fat diet. *J. Oleo Sci.,* 55, 79–89 (2006).

16) Akazome, Y., Kanda, T., Ohtake, Y., Hashimoto, H., Kametani, N., Sato, K., Nakamura, T., Kajimoto, Y., Evaluation of safety of excessive intake and efficacy of long-term intake of beverage containing polyphenols derived from apples. *Jpn. Pharmacol. Ther.,* 33, 893–911 (2005).

17) Leontowicz, H., Leontowicz, M., Gorinstein, S., Lojek, A., Ciz, M., Soliva-Fortuny, R., Martin-Belloso, O., Jung, S.T., Trakhtenberg, S., Comparative content of some bioactive compounds in apples, peaches, and pears and their influence on lipids and antioxidant capacity in rats. *J. Nutr. Biochem.,* 13, 603–610 (2002).

18) Schaefer, S., Baum, M., Eisenbrand, G., Janzowski, C., Modulation of oxidative cell damage by reconstituted mixtures of phenolic apple juice extracts in human colon cell lines. *Mol. Nutr. Food Res.,* 50, 413–417 (2006).

19) Lapidot, T., Kanner, J., Walker, M.D., Can apple antioxidants inhibit tumor cell proliferation? Generation of H_2O_2 during interaction of phenolic compounds with cell culture media. *J. Agric. Food Chem.,* 50, 3156–3160 (2002).

20) Gossé, F., Guyot, S., Roussi, S., Lobstein, A., Fischer, B., Seiler, N., Raul, F., Chemopreventive properties of apple procyanidins on human colon cancerderived metastatic SW620 cells and in a rat model of colon carcinogenesis. *Carcinogenesis,* 26, 1291–1295 (2005).

21) Hibasami, H., Shoji, T., Shibuya, I., Higo, K., Kanda, T., Induction of apoptosis by three types of procyanidin isolated from apple (*Rosaceae Malus pumila*) in human stomach cancer KATO III cells. *Int. J. Mol. Med.,* 13, 795–799 (2004).

22) Miura, T., Chiba, M., Kasai, K., Nozaka, H., Nakamura, T., Shoji, T., Kanda, T., Ohtake, Y., Sato,

T., Apple procyanidins induce tumor cell apoptosis through mitochondrial pathway activation of caspase-3. *Carcinogenesis,* 29, 585–593 (2008).

23) Sugiyama, H., Akazome, Y., Shoji, T., Yamaguchi, A., Yasue, M., Kanda, T., Ohtake, Y., Oligomeric procyanidins in apple polyphenol are main active components for inhibition of pancreatic lipase and triglyceride absorption. *J. Agric. Food Chem.,* 55, 4604–4609 (2007).

24) Ogino, Y., Osada, K., Nakamura, S., Ohta, Y., Kanda, T., Sugano, M., Absorption of dietary cholesterol oxidation products and their downstream metabolic effects are reduced by dietary apple polyphenols. *Lipids,* 42, 151–161 (2007).

25) Sunagawa, T., Shimizu, T., Kanda, T., Tagashira, M., Sami, M., Shirasawa, T., Procyanidins from apples (*Malus pumila Mill*.) extend the lifespan of *Caenorhabditis elegans*. *Planta. Med.,* 77, 122–127 (2011).

26) Shoji, T., Akazome, Y., Kanda, T., Ikeda, M., The toxicology and safety of apple polyphenol extract. *Food Chem. Toxicol.,* 42, 959–967 (2004).

27) Wren, A.F., Cleary, M., Frantz, C., Melton, S., Norris, L., 90-Day oral toxicity study of a grape seed extract (IH636) in rats. *J. Agric. Food Chem.,* 50, 2180–2192 (2002).

28) Yamakoshi, J., Saito, M., Kataoka, S., Kikuchi, M., Safety evaluation of proanthocyanidin-rich extract from grape seeds. *Food Chem. Toxicol.*, 40, 599–607 (2002).

29) Bentivegna, S.S., Whitney, K.M., Subchronic 3-month oral toxicity study of grape seed and grape skin extracts. *Food Chem. Toxicol.,* 40, 1731–1743 (2002).

30) Takami, S., Imai, T., Hasumura, M., Cho, Y.M., Onose, J., Hirose, M., Evaluation of toxicity of green tea catechins with 90-day dietary administration to F344 rats. *Food Chem. Toxicol.,* 46, 2224–2229 (2008).

31) Jin, M., Muguruma, M., Moto, M., Okamura, M., Kashida, Y., Mitsumori, K., Thirteen-week repeated dose toxicity of Siraitia grosvenori extract in Wistar Hannover (GALAS) rats. *Food Chem. Toxicol.,* 45, 1231–1237 (2007).

32) Goldsmith, L.A., Acute and subchronic toxicity of sucralose. *Food Chem.Toxicol.,* 38, 53–69

(2000).

33) Casterton, P.L., Curry, L.L., Lina, B.A., Wolterbeek, A.P., Kruger, C.L., 90-Day feeding and genotoxicity studies on a refined arachidonic acid-rich oil. *Food Chem. Toxicol.,* 47, 2407–2418 (2009)..

34) Shoji, T., Masumoto, S., Moriichi, N., Akiyama, H., Kanda, T., Ohtake, Y., Goda, Y., Apple procyanidin oligomers absorption in rats after oral administration: analysis of procyanidins in plasma using the porter method and high-performance liquid chromatography/tandem mass spectrometry. *J. Agric. Food Chem.*, 54, 884–892 (2006).

35) Shahidi, F., Naczk, M., Food Phenolics. Technomic Publishing Company, Inc., Switzerland, pp. 109–115 (1995).

36) Fuleki, T., Ricardo-da-Silva, J.M., Catechin and procyanidin composition of seeds from grape cultivars grown in Ontario. *J. Agri. Food Chem.,* 45, 1156–1160 (1997).

37) Krueger, C.G., Dopke, N.C., Treichel, P.M., Folts, J., Reed, D., Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of polygalloyl polyflavan-3-ols in grape seed extract. *J. Agric. Food Chem.,* 48, 1863–1867 (2000).

38) Santos-Buelga, C., Francia-Aricha, E.M., Escribano-Bailon, M.T., Comparative flavan-3-ol composition of seeds from different grape varieties. *Food Chem.,* 53, 197–201 (1995).

39) Tada, Y., Yano, N., Takahashi, H., Yuzawa, K., Ando, H., Kubo, Y., Nagasawa, A., Uehara, S., Ogata, A., Nakae, D., Toxic effects of L-aspartic acid at high dose levels on kidneys and salivary glands in Fischer 344 rats detected in a 90-day feeding study. *Food Chem. Toxicol.,* 46, 2789–2795 (2008).

40) Karaman, S., Barnett Jr., J., Sykes, G.P., Delaney, B., Subchronic oral toxicity assessment of N-acetyl-L-aspartic acid in rats. *Food Chem. Toxicol.,* 49, 155–165 (2011).

41) Buchner, A., Sreebny, L.M., Enlargement of salivary glands. *Oral Surg.* 34, 209–222 (1972).

42) Burdock, G.A., Flamm, W.G., Carabin, I.G., Toxicity and mutagenicity studies of DN-50000® and RP-1® enzymes. *Food Chem. Toxicol*., 38, 429–442 (2000).

43) Wells, H., Voelkel, E.F., Submandibular salivary gland enlargement by feedingpancreatin to rats. *Am. J. Physiol.*, 205, 1117–1121 (1963).

44) Wells, H., Peronace, A.A.V., Functional hyper-trophy and atrophy of the salivary glands of rats. *Am. J. Physiol.*, 212, 247–251 (1966).

45) Tamano, S., Kawabe, M., Hatahara, Y., Sano, M., Hagiwara, A., Fukumoto, T., Nakamura, M., Shirai, T., 13-Week oral toxicity of guarana extract in F344/ DuCrj rats. Jpn. *J. Food Chem.*, 12, 63–69 (2005).

46) Tarka Jr., S.M., The toxicology of cocoa and methylxanthines: a review of the literature. *Crit. Rev. Toxicol.*, 9, 275–312 (1982).

Summary

Chapter 1

We examined the effects of (－)-Hydroxycitrate (HCA) ingestion on fat oxidation during moderate intensity exercise in untrained men. Six subjects ingested 500 mg of HCA or a placebo for 5 days and did endurance exercise. Blood FFA concentrations were significantly increased and respiratory exchange ratio (RER) decreased by HCA ingestion. These results suggested short-term HCA ingestion increases fat oxidation in untrained men.

Chapter 2

We examined the effects of a nondigestible disaccharide difructose anhydride III (DFAIII) on calcium absorption and retention by means of a human balance study of single-blind crossover design. Twelve healthy male subjects ingested 250mg of shell powder as calcium carbonate (corresponding to 100mg of calcium) with or without 1.0 g DFAIII three times a day for 13 d. In the last 4 d as a balance period, all urine and feces were collected and evaluated for calcium excretion. The apparent calcium absorption (mg/d) and rate of absorption (%) were higher, and those of retention were much higher, in the DFAIII group than in the control group. Furthermore, serum osteocalcin increased after the experimental period in the DFAIII group but not in the control group. These results indicate that DFAIII ingestion enhances intestinal calcium absorption, which might be beneficial for bone metabolism.

Chapter 3

We examined how dietary melibiose affected the Thelper (Th) cell responses induced by an orally fed antigen in ovalbumin (OVA)-specific T cell receptor transgenic mice (OVA 23-3). Dietary melibiose markedly decreased the Th2 type responses as shown by a significant decrease in the interleukin (IL)-4 production and T cell proliferative response induced by sensitization from the 7-d

oral administration of OVA. It was additionally observed that the Th1 type responses tended to decrease. We therefore examined the effect of melibiose feeding on the induction of immunological tolerance induced by the oral administration of an antigen (oral tolerance). The Th cell responses induced in BALB/c mice by subcutaneous immunization with OVA were suppressed by the prior oral administration of OVA. Such responses in the OVA-fed and immunized mice were further diminished by dietary melibiose. These results suggest that dietary melibiose strongly affected the Th cell responses to an ingested antigen, and further demonstrate the potential of melibiose to enhance the induction of oral tolerance.

Chapter 4

To examine the safety of dietary Applephenon® (AP) in feed, Crl: CD (SD) rats of each sex were divided into four groups and given diets containing AP at 0%, 1.25%, 2.5%, or 5.0% for 90 days. All rats survived and toxic changes were not observed throughout the study. Body weight and food efficiency in the 5.0% AP group of both sexes were significantly decreased compared with that in controls. These changes were considered to be caused by the physiological effects of AP (including the inhibitory effects on pancreatic lipase activity). Slight hypertrophy in acinar cells in the parotid and submandibular glands appeared in the 2.5% and 5.0% groups. These were suggested not to be toxicological but physiologic adaptive responses to oral stimuli by the lower pH of AP-containing diets. In conclusion, dietary AP in feed, up to a maximum level of 5.0% for 90 days, given to rats did not induce toxicological effects.

Acknowledgements

The author would like to express her sincere gratitude to Dr. Teruo Kawada, Professor of Graduate School of Agriculture, Kyoto University, for his continuous guidance, valuable discussions, and cordial encouragement throughout the course of this study.

The author would like to express her gratefulness to Dr. Kiwon Lim, Department of Physical Education, Konkuk University, Korea, Dr. Hiroshi Hara, Professor of Graduate School of Agriculture, Hokkaido University, and Dr. Satoshi Hachimura, Associate Professor of Graduate School of Agricultural and Life Sciences, The University of Tokyo, for their helpful suggestion and valuable discussion.

The author would like to express her gratitude for Dr. Nirihiro Shigematsu and Dr. Manzhen Shen (Fancl.Co.), Dr. Taizo Nagura (Nippon Beet Sugar Manufacturing Co., Ltd.), Dr. Tomomasa Kanda and Dr. Manabu Sami (Asahi Group Holdings Ltd.), and Dr. Ryuzo Sasaki, for their helpful suggestion and cordial encouragement.

The author would like to show her greatest appreciation to all her collaborators and her family members for their continuous supports and cheer.

List of publication

1. **Kyoko Tomita**, Yasuhide Okuhara, Norihiro Shigematsu, Heajung Suh, and Kiwon Lim

(-)-Hydroxycitrate ingestion increases fat oxidation during moderate intensity exercise in untrained men.

Biosci. Biotechnol. Biochem., 67, 1999-2001, (2003)

2. **Kyoko Tomita**, Takuya Shiomi, Yasuhide Okuhara, Akiko Tamura, Norihiro Shigematsu, and Hiroshi Hara

Ingestion of difructose anhydride III enhances absorption and retention of calcium in healthy men.

Biosci. Biotechnol. Biochem., 71, 681-687, (2007)

3. **Kyoko Tomita**, Taizo Nagura, Yasuhide Okuhara, Haruyo Nakajima-Adachi, Norihiro Shigematsu, Tsutomu Aritsuka, Shuichi Kaminogawa, and Satoshi Hachimura

Dietary melibiose regulates Th cell response and enhances the induction of oral tolerance.

Biosci. Biotechnol. Biochem., 71, 2774-2780, (2007)

4. **Kyoko Fujiwara**, Shohei Nakashima, Manabu Sami and Tomomasa Kanda.

Ninety-day Dietary Toxicity Study of Apple Polyphenol Extracts in Crl: CD (SD) Rat.

Food Chem. Toxicol., 56, 214-222, (2013)